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USE OF UNICELLULAR ALGAE FOR EVALUATION OF POTENTIAL AQUATIC CONTAMINANTS SECOND ANNUAL REPORT

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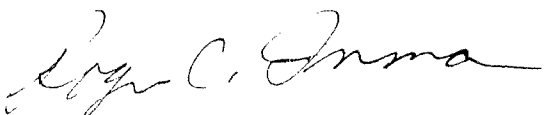

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Reference Mixture	EC50	Continuous Culture System
Relative Toxicities	MATC	Electron Microscopy

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Selenastrum capricornutum was used as test algae in bioassays to determine the No Effect Level (NOEL), Effective Concentration (EC50), and Maximum Allowable Toxic Concentration (MATC) for conventional JP-4, JP-8, Shale-Derived JP-4, and Shale-Derived JP-8 with and without clay treatment. Preliminary investigations were conducted to evaluate the relative toxicity of a reference jet fuel mixture composed of equal parts of 15 major fuel compounds. Techniques and protocols are described, and the results are discussed.

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SUMMARY

This annual report presents the results of investigations conducted at the Water Resources Laboratory of UC Irvine during 1981/82 to evaluate and quantify the relative toxicities of certain jet propellants, especially shale-derived fuels, that may be released into waters of different nutrient status during Air Force operations.

During the past decade, increasing emphasis on environmental protection has led to the evaluation of possible adverse effects of new compounds (including fuels) which are planned to be introduced for general industrial or military use. One such important group of compounds includes shale-derived fuels which may increase in importance during periods of reduced oil importation.

The results presented herein will be used by USAF/USN to determine how activities involving such compounds can be conducted in conformity with the National Environmental Policy Act, and also provide a quantitative basis for the selection of alternate fuels which may have significantly different environmental effects from conventional fuels while having equivalent combustion characteristics.

The investigations were based principally on the use of the relatively rapid and convenient batch algal bioassay procedures, as well as consideration of continuous culture methods. The initial batch algal assay provides a basis for assessing the impact of aquatic contaminants on unicellular algae. This proven excellent screening tool was then supplemented with further detailed investigations based on GC/MS of the water soluble fraction of the fuel in order to identify components of high toxicity.

CONCLUSIONS

The following conclusions can be drawn from the investigations conducted during 1981/1982:

1. Screening experiments were undertaken to determine the toxic effects of JP-4, JP-8, shale-derived JP-8 with and without clay-treatment, and shale-derived JP-4. Growth response of the test organism Selenastrum capricornutum in media exposed to fuel inoculates of various concentrations showed the following increasing toxicities:

   SDJP-4 < JP-4 < JP-8 < SDJP-8 < CTSDJP-8.

2. Bioassays using JP-8 with and without clay-treatment showed that algal growth was severely inhibited even at the lowest concentrations tested.

3. Biostimulatory effects were recorded at very low fuel concentrations for JP-4, JP-8, and SDJP-4 (1 ppm level).

4. Standard Plate Counts of media exposed to JP-4 and JP-8 indicated that the largest bacterial populations were observed with the highest fuel concentrations tested. Opposite results were obtained with shale-derived JP-8, suggesting the occurrence of bacterial inhibitory compounds in this fuel.

5. Emission spectroscopy and neutron activation techniques showed that only a fraction of 20 quantified trace elements in jet fuels passed into the water soluble fraction/emulsion; such trace elements have little or no influence in the toxic effects of these fuels.

6. A batch assay experiment was conducted on a reference jet fuel mixture composed of equal parts of 15 major fuel compounds. Results indicated that concentrations in excess of 100 ppm inhibited algal growth; biostimulatory effects were recorded for lower concentrations of the mixture.

7. Batch assays were undertaken to determine which of the reference mixture sub-groups were responsible for the overall toxicity.

   Results indicated the following ranking in toxicity:

   unsaturated rings < saturated rings < straight chains < double rings.

8. Continuous culture experiments showed that for a feed rate of 0.32 ml/min of 33% SAAM, chemostats could not support the algal population for more than 14 days. Fed with 100% SAAM, chemostats showed a progressive achievement of maximum standing crop.

INTRODUCTION

The use of the new jet propellants, additives and/or lubricants introduced by USAF/USN presents the possibility of spillage into the aquatic environment, and the effects of these compounds in various types of aquatic systems must be determined.

Phytoplankton constitutes one of the most important elements of the ecosystem in natural waters because of its role as primary producer. Unicellular algae are recommended by US-EPA for use as

bioassay organisms and the methodology for using Selenastrum capricornutum in determining growth and/or inhibitory effects has been well established. Algal bioassays may also be used to evaluate both the possibility of toxic compound bioaccumulation and mutagenesis phenomena.

OBJECTIVES

Research objectives for the second year have been directed towards the establishment of dose/concentration responses of unicellular green algae to the water soluble fraction/emulsion of jet fuel. Special emphasis was placed on the evaluation of the physico-chemical composition of the volatile, dissolved and emulsified fractions of jet fuels. Compounds studied included regular JP-4 and JP-8, shale-derived JP-4 (SDJP-4), shale-derived JP-8 (SDJP-8), and shale-derived JP-8 with clay treatment (CTSDJP-8).

Quantitative bioassays were conducted to establish dose/concentration responses of S. capricornutum in various aquatic environments, in conformance with Algal Assay Procedure (Algal Assay Procedure, 1982), and the Standard Methods for the Examination of Water and Wastewater (Standard Methods, 1975). Batch assay experiments were conducted to determine the no effect level (NOEL) of the jet fuels tested, and the toxicity of a "Comparison Mixture", the composition of which was proposed in a joint project meeting at UCI in the winter of 1981 (AFAMRL, AFESC, 1981).

Towards the end of the project year, a continuous culture system was started to determine low level dynamic chronic exposure effects. The overall goals have been to provide information about relative safety of jet fuel hydrocarbon compounds, determine threshold limits, to obtain relevant data which can be used for the engineering design of waste treatment systems, and clean-up procedures for accidental spills.

Specific Objectives

1. Determine the quantity and composition of the fraction of each fuel that enters the water under different contact conditions (which may simulate alternative ways by which fuels may enter the aquatic environment).

2. Determine the relative amounts of fuel components in true solution in the aqueous phase compared with those occurring as emulsified droplets.

3. Estimate the fate of fuels and of the fractions of their main components as a function of time in the batch algal assay. This

also provides general information about the relative stability of the components from different fuels remaining in natural environments after spills.

4. Determine the no effect level (NOEL) for each of the jet fuels tested under the various test conditions.

5. Determine the maximum allowable toxic concentration (MATC), and median effective concentration (EC_{50}) for each of the jet fuels tested under the various test conditions.

6. Assess the relative toxicity of trace metals in the water soluble fraction under bioassay conditions.

7. Test and evaluate an artificial 15-component fuel mixture (containing the same main groups of organic compounds as regular fuels), and determine the effect and fate of each group or compound under algal assay conditions. Although such an artificial mix will not exactly resemble an actual fuel, it can be used to assure that different laboratories obtain similar assay results.

8. Determine equilibrium parameters for continuous culture chemostats as a function of feed rate, growth medium strength, washout rate and time; and then begin chronic exposure evaluations.

9. Prepare a summary batch assay protocol which can be used as part of fuel specifications to verify that different batches purchased by the Air Force do not have significantly higher environmental effects from a "prototype fuel".

WORKPLAN

The workplan was divided into three main parts related to the specific objectives:

1. Completion of NOEL, EC_{50} and MATC determinations for JP-4, JP-8, SDJP-4, SDJP-8 and CTSDJP-8, initiated during the 1980/81 research period.

2. Develop analytical procedures to investigate the stability and composition of the WSF/E of the comparison mixture under various experimental conditions.

3. Initiate continuous culture experiments to quantify in simulated natural conditions the response of aquatic ecosystems under chronic exposure to jet fuels.

TOXICITY MEASUREMENTS AND TEST METHODS

TOXICITY MEASUREMENTS

Two key approaches were used in this work to form the basis for the conclusions regarding the effects of the Water Soluble Fraction/Emulsion (WSF/E) of jet fuels in the aquatic environment:

Biological Growth Measures

The main concept used was the measure of biological activity. During the early periods of this investigation, extensive work was done to evaluate the applicability and methods to interpret the results obtained with various parameters including but not limited to oxygen production rates, specific growth rates, and maximum biomass produced (Scherfig et al., 1977). Based on that work it was decided that two measures would be used to evaluate the effects of the WSF/E of jet fuels.

The first measure is the maximum standing crop, which is considered to have been reached when the increase in algal growth is less than five percent per day. One major difficulty encountered with this measure is related to the instability of WSF/E of jet fuels compared with the normal 10-15 days required to reach maximum standing crop. In order to determine the absolute and relative toxicity of the WSF/E compounds, it was decided to quantify the effects by relative growth compared with a control sample after six, eight and ten days of growth. The relative growth figures were then compiled to determine the toxic concentrations of the jet fuels' WSF/E.

Toxic Concentrations

Three complementary measures were selected to quantify the toxic levels of jet fuel compounds.

The first of these is the NOEL, which is the maximum concentration of any jet fuel or comparison mixture (CM) which can be present without causing a statistically detectable difference in the maximum standing crop.

The second measure used was the maximum allowable toxic concentration (MATC), which is that concentration resulting in a ten percent reduction in algal growth on the sixth day compared with the control.

The third measure used was the median effective concentration (EC_{50}), which is that concentration resulting in a 50 percent reduction in algal growth on the sixth day of growth compared with the control.

TEST METHODS

Algal Bioassays

Algal bioassays were conducted in accordance with Algal Assay Procedure (Algal Assay Procedure, 1981) and Standard Methods (Standard Methods, 1975) to determine jet fuel and CM WSF/E NOEL, EC_{50}, and MATC under various experimental conditions.

Modifications of the Algal Assay Procedure included the following:

1. A larger volume of medium was used (250 ml/500 ml flasks), but this was shown to require no change in the auxiliary aeration system used.

2. Temperature control was 25 ± 1° C.

3. All compounds contained in the growth medium were added in a specified order before filtration in order to prevent iron precipitation. The sequence was sodium bicarbonate, magnesium sulfate, calcium chloride, potassium orthophosphate (mono-H), magnesium chloride, sodium nitrate and the trace metals including a chelating agent.

Algal bioassays were conducted in two steps. The first was a broad screening test. This was followed by a second and more detailed evaluation analysis. Initially, a preliminary series of replicate flasks containing the algal growth medium was dosed with a broad range of concentrations (from 0.05 to 1000 ppm) of the test compound.

Flasks were then seeded with S. capricornutum, and algal growth (both total cell and total algal volume) was monitored with an electronic particle counter (Coulter Counter model TA II, with population accessory) until at least the control flasks reached the maximum standing crop. This allowed a preliminary approximation of the NOEL and EC_{50} concentration ranges. Another series of flasks containing Standard Algal Assay Medium (SAAM) was then dosed with concentrations of the test compound within the preliminary range. All flasks were seeded with S. capricornutum to an initial concentration of 10^6 cells/ℓ. Algal growth was monitored as described above and the NOEL, EC_{50} and/or MATC were determined by

statistical procedures as described in Standard Methods (Standard Methods, 1975).

Test compounds were freshly prepared by serial dilution from the stock bottle immediately before being added to the bioassay flasks seeded with algal cells. At least five replicate flasks were prepared for each of the desired initial concentrations of test compound. Total carbon (TC) concentrations were checked in each flask at the beginning of each experimental run. In most cases, the desired and actual initial test compound concentrations were in agreement.

Continuous Culture System

During the second half of the project year, major emphasis was placed on the establishment of an automated continuous culture system. Following preliminary testing, best results were obtained with the following components:

1. Two stands of four glass tubular chemostats were located on each side of a refrigerator. Each 23" high and 2" I.D. vessel (capacity of 920 ml) was clamped vertically on the frame, and had an overflow port towards the top, and a dual aeration/growth medium feed port towards the bottom.

2. Individual vessels had continuous illumination at 350 ± 50 foot candles with cool white fluorescent tubes.

3. Homogeneous mixing of the contents of each chemostat was ensured by a teflon magnetic stirring bar set at 250 ± revolutions/ minute.

4. The compressed air supply was first filtered to strip residual oil mist and then filtered through sterilized 0.45 micrometer Millipore filter pads. Air flows were individually adjusted to 350 ± 50 ml/min.

5. Each chemostat was continuously fed with the appropriate growth medium, using a multi-channel Technicon pump.

Both the medium stock solution and the overflow were maintained inside the refrigerator at 4° C. The feed and overflow lines were connected through the side panels of the refrigerator for each stand of four chemostats, from the SAAM stock to the pump, and from the overflow to sampling flasks. Flow-rated according to their I.D., the pumping tubes could be changed to permit variation of the residence times for individual tubes.

All glassware and tubing was carefully sterilized with a chlorine solution and then rinsed with deionized water prior to each experimental run. The SAAM preparation procedures were also modified to include the addition of 6 mg/ℓ of penicillin following final filtration. This was done to control bacterial contamination in the transfer lines.

The chemostats were monitored for major variables such as illumination, mixing, temperature, pH, total carbon, nitrogen, phosphorus, iron concentration, and feed rates.

Selection of Test Compound Concentration

Dose levels were chosen to simulate the range of possible spillage conditions that may occur in conjunction with USAF/UN operations.

To simulate such conditions effectively, batch cultures were exposed to various single concentrations of each test agent corresponding to levels which may be encountered in different water bodies with different spill conditions. Specific levels of fuel dosage ranged from 0.05 ppm to 1000 ppm.

Two major methods for testing each fuel were used for the evaluation of the toxicity of the WSF/E: WSF/E alone and WSF/E with fuel layer on the surface of media containing the test organisms. The decision to first evaluate the WSF/E compounds alone was based on the assumption that only the WSF/E of the contaminants became fully incorporated in the water medium and was taken up by the biological systems within the medium. The sampling schedule was designed specifically to maintain the closest possible monitoring of critical determining variables. An example of an important sampling period relates to the critical days of the algal growth cycle, occurring on days 4 through 10, when nutrient and, possibly, test agent uptakes are at a maximum level.

Measuring growth characteristics in terms of cell number and cell volume is essential for quantitative determination of toxic effects. In addition, periodic determinations of assay medium composition throughout the test period are necessary to detect eventual changes in chemical make-up. Fuel stability constitutes one of the major areas of concern in toxicologic investigations. Previous research showed highly significant and rapid decomposition rates for some non-hydrocarbon fuels, suggesting three possible mechanisms of fuel decomposition:

1. Alteration through selective evaporation into the atmosphere.

2. Alteration through chemical reactions with certain chemical components passing into the test medium.

3. Reduction through biological utilization by algal and/or bacteria present in the test medium.

To determine whether any or all of these mechanisms were significant, monitoring protocols were established to measure the amount of test agent in the medium throughout the test while exposing the agent to these potentially altering processes.

Analytical Procedures

--Coulter Counter Determinations

Counts were performed with a Coulter TA II Particle Counter to determine algal cell number and cell volume on growth days 6, 8, and 10 in the bioassay flasks initially seeded with S. capricornutum. Results of Coulter channels 6, 7, and 8 (most representative of a typical algal cell size range) were analyzed statistically to determine NOEL and EC_{50}. The Coulter Counter was also used to investigate the fuels' emulsion droplet size greater than 0.22 µm, which was its limit of resolution.

Each concentration of the various fuels in 33% SAAM was prepared with five replicates. Each experimental flask was seeded with S. capricornutum at 10^6 cells/ℓ. Initial contamination of cells by direct contact with the fuel was avoided by seeding the experimental flask before the test fuel was added to the assay medium (direct fuel layering avoiding any initial mixing of the two phases). Prior to the addition of the fuel, a sampling tube was introduced into each flask, allowing sampling of the water phase on days 6, 8, and 10. Each flask was covered to prevent contamination, and was aerated continuously throughout the experiment. Assay flasks were maintained on lighted shaker tables rotating at 100 rpm, at 25 ± 1°C through day 10. Particle number and values were counted on days 6, 8, and 10 after seeding, using the Coulter counter.

--Optical Microscopy Procedures

Optical microscopy evaluations were occasionally conducted to check the presence and stability of emulsified droplets and/or bacteria in the water phase after the addition of fuel to the growth medium. All samples were examined under identical conditions with a Leitz Ortholux microscope (resolution: 0.3 µm). Observations were carried out on day 8 and "particle" sizes were determined using standard microscopy scales.

--Bacteriological Counts

Standard bacteriological plate counts were performed to determine whether significant bacterial populations occurred in the experimental growth media. Petri dishes were loaded with growth media after serial dilutions ranging from 1 to 1/10,000, according to Standard Methods (2). Sterile inoculations were undertaken with medium plus algae on the sixth day following the experimental flasks' initial seeding (10^6 cells/liter). Petri dishes were incubated at a temperature of 25.5 ± 1°C, in a Brunswick incubator; bacterial colonies were counted with a Quebec colony counter 24 and 48 hours following incubation.

Further identification of bacteria isolated in pure cultures was performed at the Orange County Health Department (Santa Ana, California).

--Total Carbon Analysis

Investigations were conducted to determine the portions of test compound that were dissolved and/or emulsified into the growth medium. To determine this, samples taken from the experimental system were analyzed for fuel content. The TC concentration of the samples was used as an indirect measurement of the fuel content. Since the SAAM medium initially contained no significant amount of carbon and no other process could substantially introduce carbon into the sample, the use of this indicator was reasonable.

--Gas Chromatographic Investigations

Gas chromatographic analyses of the WSF/E for different types of fuel were performed with a Varian model 3700 gas chromatograph to provide preliminary identification of the hydrocarbon content. Procedures included solvent extractions and incorporation of predetermined tracers. For additional sensitivity and accuracy, use was made of the purge and trap system devised during the research period 1980/81 (see AFAMRL-TR-81-79; Scherfig et al., 1981).

--Phosphorus and Nitrogen Analyses

Monitoring of the phosphorus and nitrogen levels in the continuous culture system was achieved with the automated Technicon Auto-analyzer system. Concentrations of phosphorus and nitrogen were determined using colorimetric methods with wavelengths of 990 nm and 540 nm, respectively.

--Trace Element Determinations

Raw fuels and WSF/E of experimental flasks were analyzed for trace elements using emission spectroscopy and neutron activation analyses. Emission spectroscopy was conducted in coordination with West Coast Technical Services (Cerritos, California), while neutron activation analyses were performed at UCI, using the nuclear reactor managed by the School of Physical Sciences. For a discussion of the theory and procedures of these two techniques, see Pinta (Pinta, 1978).

RESULTS AND DISCUSSION

JET FUEL BIOASSAYS, NOEL AND EC_{50}

A comprehensive series of experiments was conducted on JP-4, JP-8, SDJP-8, CTSDJP-8 and SDJP-4 to determine simultaneously:

1. The amount of hydrocarbon going into solution/emulsion in several different concentrations of each fuel in 33% SAAM.

2. The NOEL, EC_{50}, and MATC for S. capricornutum, using probit analyses. Effects of each fuel were monitored on days 6, 8, and 10, with particle counting and TC quantification.

Fuel concentrations tested were respectively 10, 1, 0.1 and 0.05 ppm for both SDJP-8 and CTSDJP-8; 5000, 1000, 500 and 100 ppm for both JP-4 and JP-8; and 500, 100, 10, 1 and 0.05 ppm for SDJP-4.

Investigations carried out on SDJP-4 were not as extensive as for the remaining fuels because this fuel was not made available for study until the later part of the project year.

Algal Assay Results

Regular JP-4 and JP-8

Results of algal growth response to JP-4 and JP-8 are summarized in Tables 1 and 2 for each test flask for days 6, 8, and 10. At fuel ratios of 5000 and 1000 ppm, both fuels were effective algaecides, causing a reduction of more than 95 percent of normal growth (controls) through day 10.

TABLE 1. EFFECT OF JP-4 ON S. CAPRICORNUTUM

Measured by cell number, cell volume, and total carbon in the assay medium.

Fuel Concent. ppm		DAY 6 Num[a]	Vol[b]	TC[c]	DAY 8 Num	Vol	TC	DAY 10 Num	Vol	TC
100	x	831.0	48.5	23.6	1307.2	86.4	30.0	1418.0	98.5	34.1
	s	205.3	8.6	3.8	368.9	27.1	3.2	127.3	10.0	6.7
500	x	205.6	17.8	13.8	287.3	16.9	16.9	229.1	18.2	20.9
	s	114.6	11.6	0.9	109.3	9.1	4.9	43.1	2.5	6.4
1000	x	37.6	2.2	11.0	33.7	1.7	12.3	24.9	0.9	10.9
	s	23.9	2.3	2.1	24.6	2.3	1.4	8.9	1.0	0.9
5000	x	32.6	1.2	18.5	37.2	<0.7	21.0	38.7	<0.7	22.9
	s	8.8	1.0	2.6	9.1	0.0	1.0	8.2	0.0	3.0
control	x	1346.0	74.8	22.8	2069.0	121.0	34.7	1805	113.5	31.1
	s	330.3	16.9	4.7	382.0	14.9	5.0	375	25.2	8.1

(a) Cell number: 10^6 cells/liter
(b) Cell volume: mm3/liter
(c) Total Carbon: mg/liter
(x) Mean of five replicates
(s) Standard Deviation

TABLE 2. EFFECT OF JP-8 ON S. CAPRICORNUTUM

Measured by cell number, cell volume, and total carbon in the assay medium.

Fuel Concent. ppm		DAY 6 Num[a]	Vol[b]	TC[c]	DAY 8 Num	Vol	TC	DAY 10 Num	Vol	TC
100	x	1100	60.6	21	1328	79.2	30.7	1244	82	31.2
	s	134.9	10.6	4.8	252.9	14.9	3.4	432.1	26.3	3.6
500	x	239.1	13.3	12.6	174.9	11.6	15.9	194.6	15.1	18.2
	s	220.9	12.3	3.8	79.4	6.9	5.7	78.5	6.9	6.7
1000	x	12.3	<0.7	10.5	14.2	0.7	13	18.1	0.7	13.3
	s	1.5	0	1	6.3	0.3	1.9	7	0.3	1.2
5000	x	58.6	3.5	21.4	60.6	1	25.5	63.9	2.4	32.6
	s	8.6	0.8	2.5	4.2	0.7	1.7	34.4	2.6	2.8
control	x	1346	74.8	22.8	2069	121	34.7	1805	113.5	31.1
	s	330.3	16.9	4.7	382	14.9	5	375	25.2	8.1

(a) Cell number: 10^6 cells/liter
(b) Cell volume: mm3/liter
(c) Total Carbon: mg/liter
(x) Mean
(s) Standard Deviation

Substantial inhibition was indicated at fuel ratios of 500 ppm, reducing total cell volume by more than 70 percent for both fuel types. A partial growth inhibition of 65 and 80 percent relative to controls occurred at a 100 ppm concentration for JP-4 and JP-8 respectively.

The data presented in Tables 1 and 2 were compiled to determine the NOEL and EC_{50}, using probit analyses, as described in "Standard Methods for the Examination of Water and Wastewater" (Standard Methods, 1975). Day 6 EC_{50} levels of 160 ppm for JP-4, and 260 ppm for JP-8 indicated similar toxic effects of both fuels, with JP-4 being slightly more toxic. Interpolation of the probit curves revealed a six-day NOEL of 32 ppm for JP-4 and 79 ppm for JP-8, indicating also a slightly greater toxic potential for JP-4. Statistical confidence levels of the correctness of fit for both

probit curves were calculated to be 95 percent for JP-4 and 82 percent for JP-8.

Total carbon showed a general trend for all samples to increase in level of total carbon as a function of time. Both Tables 1 and 2 indicate a good correlation between the highest TC levels reached at 192 hours for a 100 ppm fuel concentration (slightly above NOEL) and the largest measured cell number and volumes. These results are to be expected assuming that the total carbon represents the organic carbon resulting from the absorption and subsequent release by the algae.

Higher fuel concentrations produced more complex results which cannot be readily explained. Specifically, at the high fuel ratios where no algal growth occurred, there can be no net absorption of organic carbon in the test system. The increase in TC as a function of time can then best be explained by the combination or conversion of some initially non-soluble or non-emulsified fuel components into both bacterial biomass and/or soluble/emulsifiable compounds. At the highest fuel concentration where no algal growth was recorded, the highest TC increase led to the suggestion of significant bacterial effects. This was investigated and the results which verified the hypothesis are described in a later section of this report.

Shale-derived JP-8 with and without clay-treatment

Preliminary screening experiments were conducted on SDJP-8 and CTSDJP-8 to determine the amount of hydrocarbon going into solution/emulsion under several fuel/33% SAAM ratios. Concentrations tested were 5000, 1000, 500 and 100 ppm.

Results shown in Table 3 indicated that even at the 100 ppm level, algal growth was severely inhibited, with day 6 relative growth rates of 16.4 and 18.6 percent for SDJP-8 and CTSDJP-8 respectively. Relative growth rates increased only marginally to 24.3 and 30.8 percent for SDJP-8 and CTSDJP-8 by day 10.

SDJP-8 and CTSDJP-8 were found similar in toxic effects toward algal growth, and both were much more inhibitory to algae than JP-4 or JP-8. To quantify the degree of toxicity of the shale-derived fuels, additional screening bioassays were conducted to determine the appropriate fuel concentration range over which algal growth response could be measured.

The results of the growth response of S. capricornutum exposed to 10, 1, 0.1, and 0.05 ppm of SDJP-8 and CTSDJP-8 are presented for days 6, 8 and 10 in Tables 4 and 5. No significant differences in growth were detected for either fuel at any concentration. At least two hypotheses could explain these unexpected results:

TABLE 3. RELATIVE GROWTH RESPONSE OF S. CAPRICORNUTUM BY TOTAL CELL VOLUME TO SHALE-DERIVED JP-8 AND CLAY-TREATED SHALE-DERIVED JP-8

FUEL	CONCENTRATION (ppm)	DAY 6 Mean	DAY 6 S.D.	DAY 8 Mean	DAY 8 S.D.	DAY 10 Mean	DAY 10 S.D.
SDJP-8	5000	3.4	4.7	2.7	3.7	3.0	2.8
	1000	1.5	2.2	<0.6	0.6	<0.6	0.6
	500	2.3	2.3	1.8	2.0	<1.7	1.7
	100	16.4	12.1	23.3	11.2	24.3	10.2
CTSDJP-8	5000	<0.8	0.8	3.3	2.0	3.6	3.1
	1000	<0.8	0.8	<0.6	0.6	<0.6	0.6
	500	<2.3	2.3	1.6	1.4	1.5	1.3
	100	18.6	13.7	22.6	5.2	30.8	14.3
CONTROL	0	100.0	15.3	100.0	4.7	100.0	19.2

TABLE 4. EFFECT OF SDJP-8 ON S. CAPRICORNUTUM

Measured by cell number, cell volume, and total carbon in the assay medium.

Fuel Concent. ppm		DAY 6 Num[a]	DAY 6 Vol[b]	DAY 6 TC[c]	DAY 8 Num	DAY 8 Vol	DAY 8 TC	DAY 10 Num	DAY 10 Vol	DAY 10 TC
0.05	x	1102.0	60.6	20.6	1173.0	72.3	24.8	1574.0	97.4	35.3
	s	126.5	7.7	1.7	230.3	15.5	4.1	382.4	22.7	2.3
0.10	x	986.0	56.9	23.3	1303.0	82.0	23.2	1316.0	82.8	33.0
	s	154.2	10.4	3.9	328.6	23.2	1.7	314.3	21.1	5.0
1.00	x	1102.0	63.6	24.0	1475.0	93.4	25.6	1255.0	77.6	33.2
	s	259.3	13.1	2.2	134.2	6.7	0.8	302.4	13.7	1.8
10.00	x	1235.0	64.7	21.1	1315.0	74.3	27.5	1425.0	84.8	31.7
	s	185.2	5.8	2.3	131.7	16.5	4.8	17.9	5.3	2.1
control	x	1219.0	78.8	20.2	1067.0	65.5	28.4	950.0	62.6	33.6
	s	364.8	6.4	2.9	366.6	19.4	1.9	341.2	22.5	2.4

(a) Cell number: 10^6 cells/liter
(b) Cell volume: mm^3/liter
(c) Total Carbon: mg/liter
(x) Mean of five replicates
(s) Standard Deviation

TABLE 5. EFFECT OF CTSDJP-8 ON S. CAPRICORNUTUM

Measured by cell number, cell volume, and total carbon in the assay medium.

Fuel Concent. ppm		DAY 6 Num[a]	Vol[b]	TC[c]	DAY 8 Num	Vol	TC	DAY 10 Num	Vol	TC
0.05	x	1189.0	63.0	22.9	1140.0	65.4	29.3	1297.0	74.7	33.5
	s	263.6	15.9	2.9	30.6	6.5	2.1	299.8	21.8	1.8
0.10	x	1066.0	61.6	20.1	1439.0	92.1	30.5	1334.0	80.0	36.4
	s	151.2	11.1	2.2	385.6	33.4	3.8	376.2	25.5	3.9
1.00	x	1059.0	56.1	23.5	1415.0	82.8	33.1	1193.0	66.2	32.9
	s	91.3	9.4	4.1	199.3	12.8	4.6	177.3	5.32	2.6
10.00	x	1093.0	61.0	24.0	1283.0	76.8	32.4	1173.0	69.9	33.4
	s	291.9	17.3	2.5	107.4	5.6	3.0	146.1	13.0	1.6
control	x	1219.0	78.8	20.2	1067.0	65.5	28.4	950.0	62.6	33.6
	s	364.8	6.4	2.9	366.6	19.4	1.9	341.2	22.5	2.4

(a) Cell number: 10^6 cells/liter
(b) Cell volume: mm^3/liter
(c) Total Carbon: mg/liter
(x) Mean of five replicates
(s) Standard Deviation

1. Experimental flasks were shaken continuously at 100 rpm and aerated, so that the medium plus fuel circulated slowly. Consequently, even with a 200-fold increase in fuel concentration from 0.05 to 10 ppm, the amount of fuel was so low that the growth medium was not separated from the atmosphere by a fuel layer. Furthermore, active tension forces between the fuel and the medium resulted in an accumulation of fuel on the glassware at the surface of the medium, so that the 200-fold increase in the amount of fuel was not reflected in the percent of fuel going into solution/emulsion. This hypothesis was supported by the consistency in TC values for both SDJP-8 and CTSDJP-8 at all concentrations.

2. The second hypothesis considered the effects of the algal inoculum. For both fuels, particle counts on day 6 were slightly

lower than in controls, suggesting a minor inhibiting effect, although the opposite trend was recorded on days 8 and 10. This suggested that low concentrations of both fuels had a biostimulatory effect after an initial inhibitory effect. Most likely the reverse in trend was the consequence of the disappearance of some volatile fraction.

The TC content in relation to fuel type and concentration showed a consistent pattern for both shale-derived fuels. No significant statistical difference could be detected between any concentration and the controls except on day 3, where TC values of medium plus fuels were approximately 75 percent of the controls. This inhibitory effect during the first three days of the experiment was consistent with counts of cell number and volume. That no significant differences could be detected provided additional support for the hypotheses outlined previously.

In summary, SDJP-8 and CTSDJP-8 had similar effects on algal growth, and both were more toxic than either conventional JP-4 or JP-8. At very low concentrations of SDJP-8 and CTSDJP-8, algal growth may be stimulated. For either shale-derived fuel, the NOEL was at a concentration below 0.05 ppm, while the EC_{50} lay between 10 and 40 ppm.

Received at UCI in the late spring of 1982, SDJP-4 was also subjected to comprehensive toxicity tests. The growth response of S. capricornutum to various concentrations of SDJP-4 are presented in Table 6, showing that slight biostimulatory effects were recorded for low fuel concentrations (less than 10 ppm). Cell number and volume at 500 ppm were approximately half those at the 100 ppm level, suggesting that growth inhibition took over between fuel concentrations of 100 to 500 ppm. NOEL and EC_{50} were computed using probit analyses which indicated MATC and EC_{50} values of 3.5 ppm and 550 ppm respectively. Total carbon values showed no significant differences between test flasks of the same day; a slight increase occurred as a function of time for all fuel concentrations tested and controls. Results obtained from Coulter channels 2 and 3 showed that bacterial contamination was minimal; the absence of bacterial interferences was most likely responsible for the consistency of flasks' TC content in the experimental flasks.

The results of further data reduction are presented in Table 7, indicating biostimulatory effects at low SDJP-4 concentrations.

TABLE 6. EFFECT OF SDJP-4 ON S. CAPRICORNUTUM

Measured by cell number, cell volume, and total carbon in the assay medium.

Fuel Concent. ppm		DAY 6 Num[a]	Vol[b]	TC[c]	DAY 8 Num	Vol	TC	DAY 10 Num	Vol	TC
1	x	903.4	101.8	29.7	1195.4	134.8	32.9	1497.0	181.8	36.1
	s	337.0	37.8	1.8	281.4	34.4	2.0	280.6	36.4	1.9
10	x	1067.0	126.5	30.5	1354.4	166.4	34.5	1262.0	160.4	37.9
	s	339.1	47.8	3.5	81.3	14.9	1.4	295.6	40.4	1.8
100	x	938.2	120.8	30.2	1055.0	141.0	34.9	1231.6	172.1	36.7
	s	276.8	35.9	2.4	393.5	52.9	2.0	384.4	53.8	2.0
500	x	554.0	67.1	27.2	880.8	132.9	33.4	588.0	93.8	36.0
	s	162.0	11.9	1.6	255.5	31.0	2.3	151.8	15.8	1.4
control	x	1136.0	135.7	29.8	1145.9	138.2	32.8	1252.8	161.6	37.2
	s	236.5	25.8	2.2	458.2	49.5	1.1	214.8	33.2	2.0

(a) Cell number: 10^6 cells/liter
(b) Cell volume: mm^3/liter
(c) Total Carbon: mg/liter
(x) Mean
(s) Standard Deviation

TABLE 7. DRY WEIGHTS FOR CELL VOLUME AND CELL NUMBERS COMPUTED AS PERCENTAGES OF CONTROL DATA

	Cell Number						Cell Volume					
ppm	M	S.D	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.
0.05	119.6	40.4	99.4	16.4	101.4	22.9	117.2	39.3	97.7	16.5	98.0	22.1
0.1	113.3	40.8	109.4	5.7	138.6	4.8	106.2	36.8	96.1	12.1	121.5	20.2
1.0	79.5	29.7	104.3	24.6	119.5	22.4	75.0	27.8	97.3	24.9	112.5	22.5
10	93.9	24.4	118.2	7.1	110.8	23.6	93.2	35.2	120.4	10.8	99.3	25.0
100	82.6	29.8	92.1	34.3	98.3	30.7	89.0	26.5	102.0	38.2	106.5	33.3
500	48.8	14.3	76.9	22.3	46.9	12.1	49.4	8.8	96.2	22.4	58.0	9.7

The summary of the response of S. capricornutum to jet fuels is expressed graphically in Figures 1 through 9. All measurements were obtained with the standard algal bioassay procedure, monitoring cell number and cell volume. Estimates of dry weight biomass were obtained by conversion of total cell volume.

Comparison of cell numbers which develop in cultures exposed to JP-4 and JP-8 indicated similar dose-response characteristics. With both JP-4 and JP-8 at concentrations below 70 to 80 mg/ℓ, there was significant deviation from the control, as shown in Figures 1 through 4. Partial inhibition was also observed at concentrations between 360 and 420 mg/ℓ.

The dose-response curves with SDJP-8 and CTSDJP-8, shown in Figures 5 through 8, indicated that both fuels produced minimal inhibition at concentrations below 8 mg/ℓ, with significant inhibition at 80 mg/ℓ. The two shale-derived fuels were found equally growth restrictive while at moderate dosages they were as much as ten times more growth restrictive than either JP-4 or JP-8. At higher concentrations, the difference between conventional and both SDJP-8 and CTSDJP-8 diminished. SDJP-4 had a lower MATC than either JP-4 or JP-8, but the extremely high EC_{50} ranks this fuel as the least toxic. Table 8 summarizes the toxicity comparison of all fuels tested.

TABLE 8. MAXIMUM ALLOWABLE TOXIC CONCENTRATION (MATC) AND EC_{50} FOR FIVE JET FUELS (EXPRESSED AS MG/ℓ), USING S. CAPRICORNUTUM BATCH ASSAY, DAY GROWTH 6 TO 20

	SDJP-4		JP-4		JP-8		SDJP-8		CTSDJP-8	
DAY	MATC	EC_{50}	MATC	EC_{50}	MATC	EC_{50}	MATC	EC_{50}	MATC	EC_{50}
6	3.5	550	55.4	199	31.7	122	2.9	27.4	2.1	20.1
8	--	--	46.3	147	32.5	105	10.5	56.7	3.1	34.6
10	--	--	63.5	185	53.4	165	11.4	56.5	5.7	45.8
14	--	--	47.4	196	71.7	228	3.7	23.6	1.3	15.4
20	--	--	61.0	215	78.2	226	29.8	42.8	17.7	79.8

TRACE METAL INVESTIGATIONS

The growth inhibitory effects of the jet fuels JP-4, JP-8, SDJP-8, and CTSDJP-8 to S. capricornutum under static assay conditions have been presented. As the physical and biochemical mechanisms involved in these effects are still largely unknown,

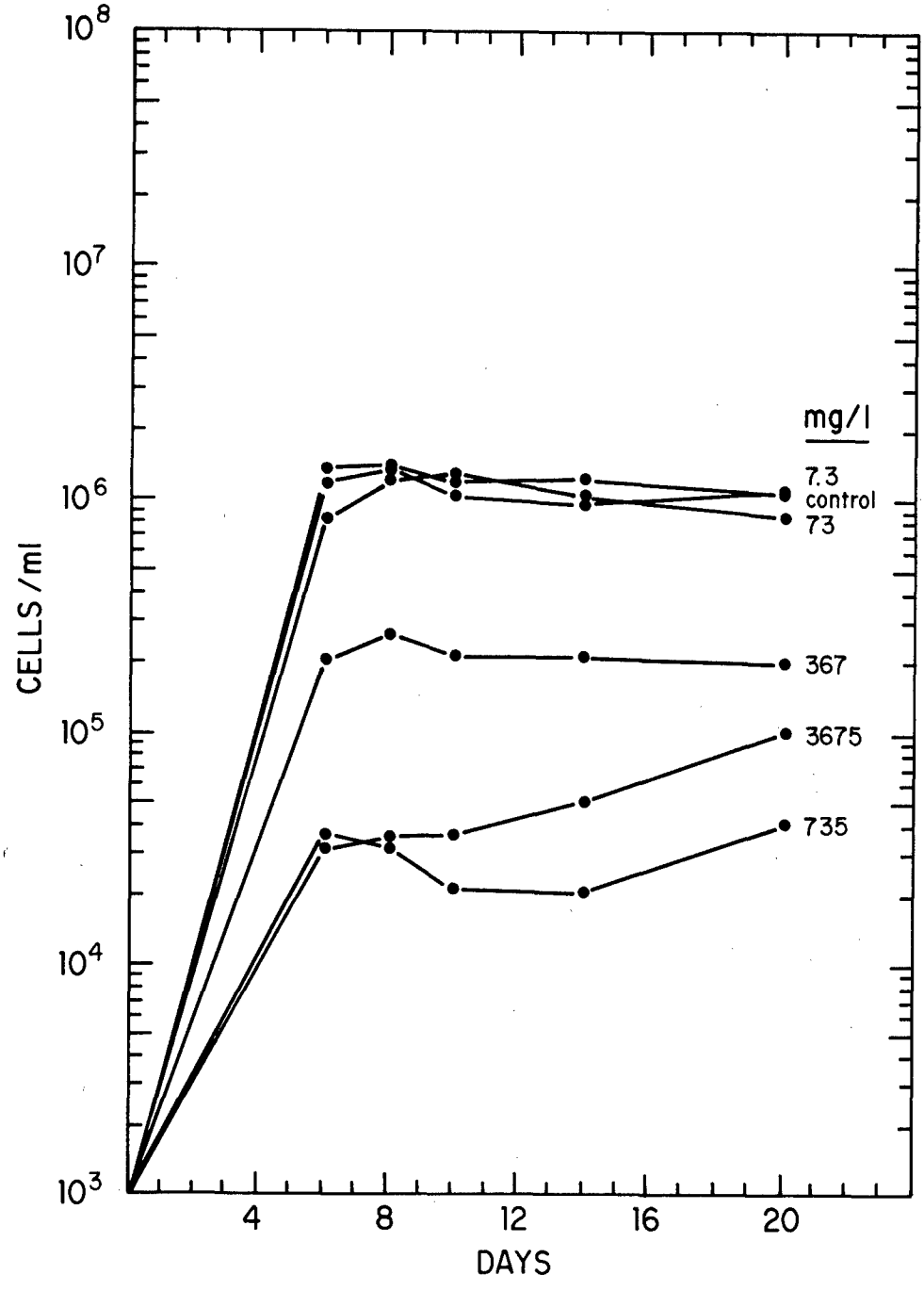

Figure 1. Growth of S. capricornutum (expressed as cells) in 33% SAAM exposed to selected concentrations of JP-4

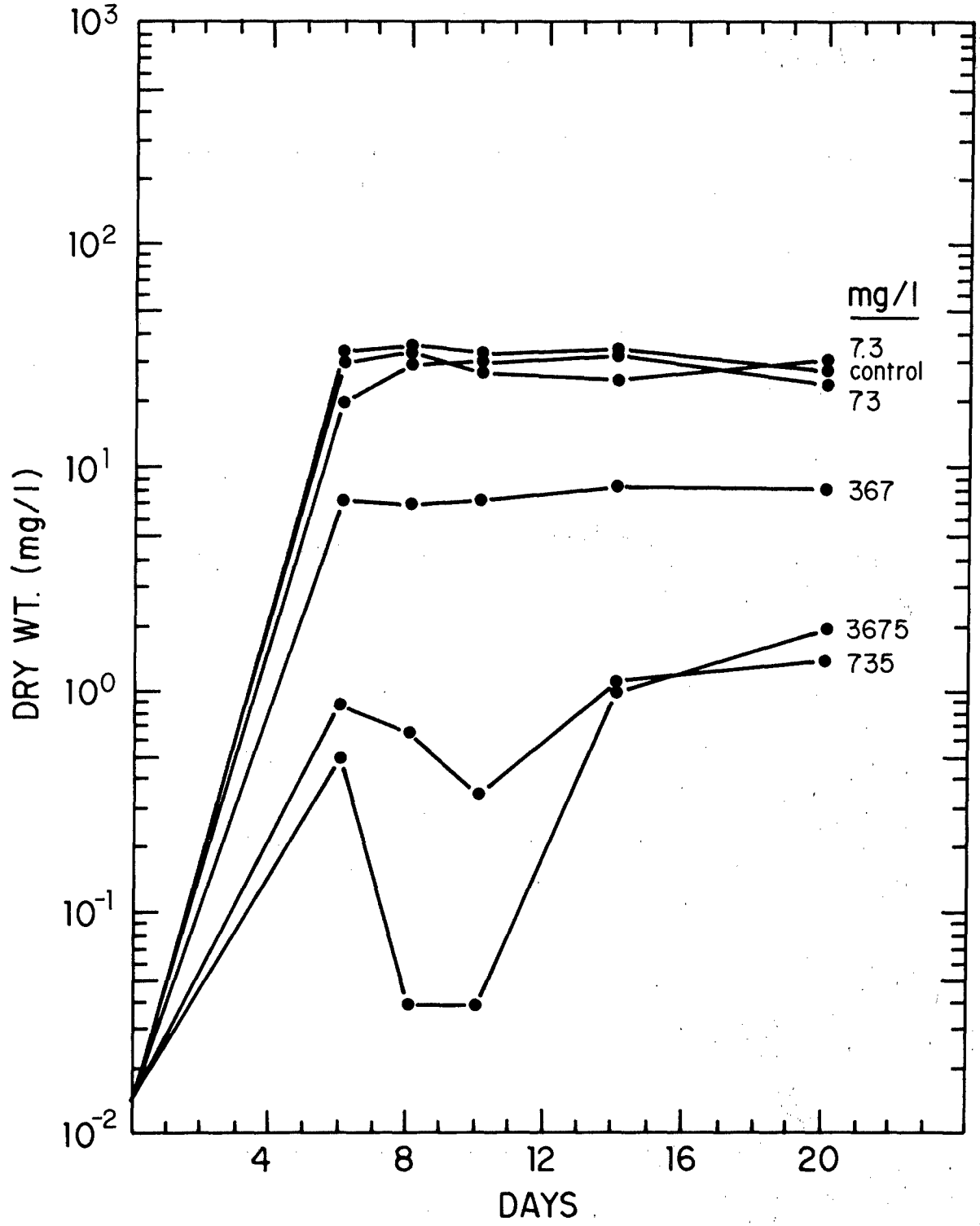

Figure 2. Growth of S. capricornutum (expressed as mg/ℓ dry weight) in 33% SAAM exposed to selected concentrations of JP-4

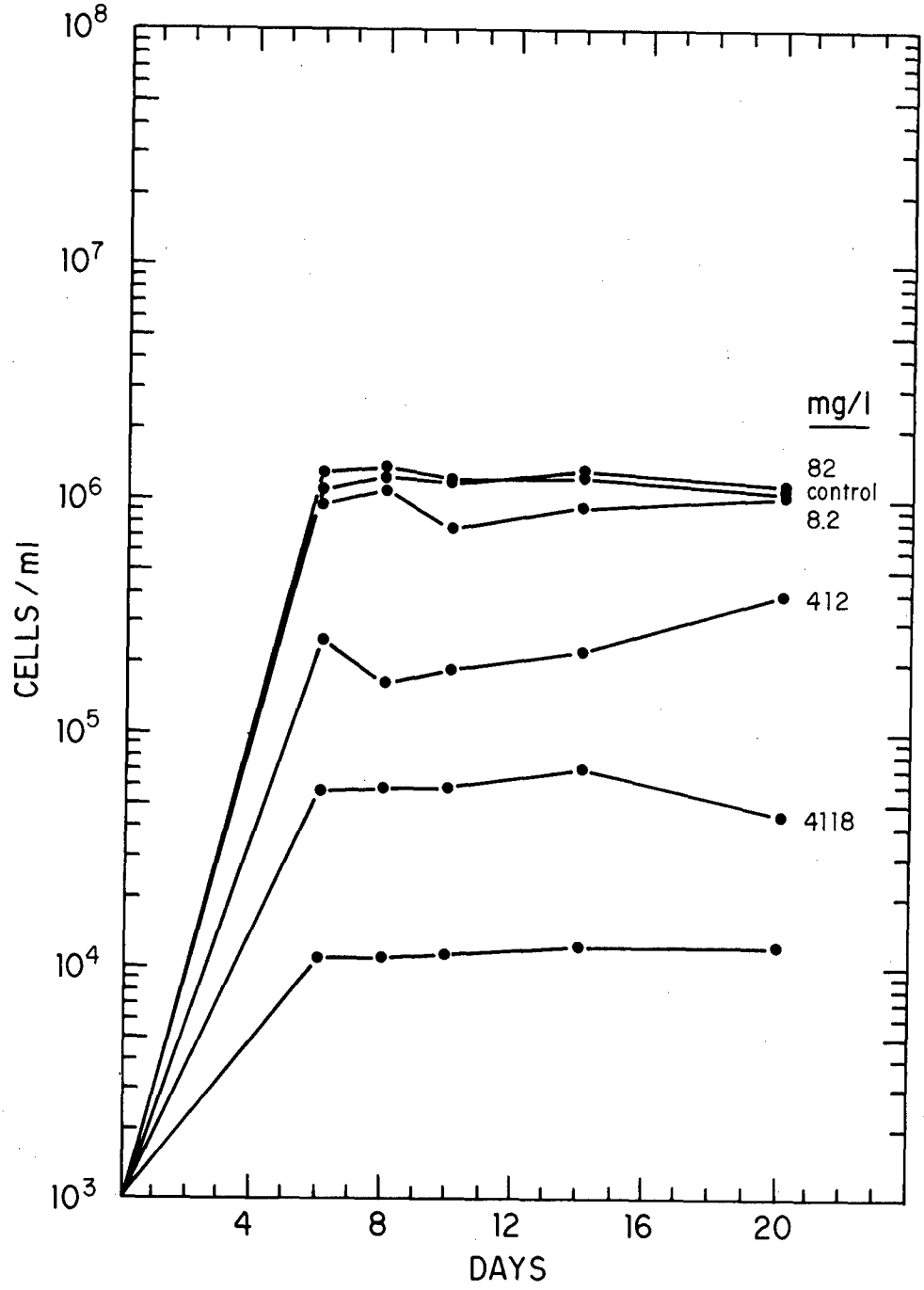

Figure 3. Growth of S. capricornutum (expressed as cells) in 33% SAAM exposed to selected concentrations of JP-8

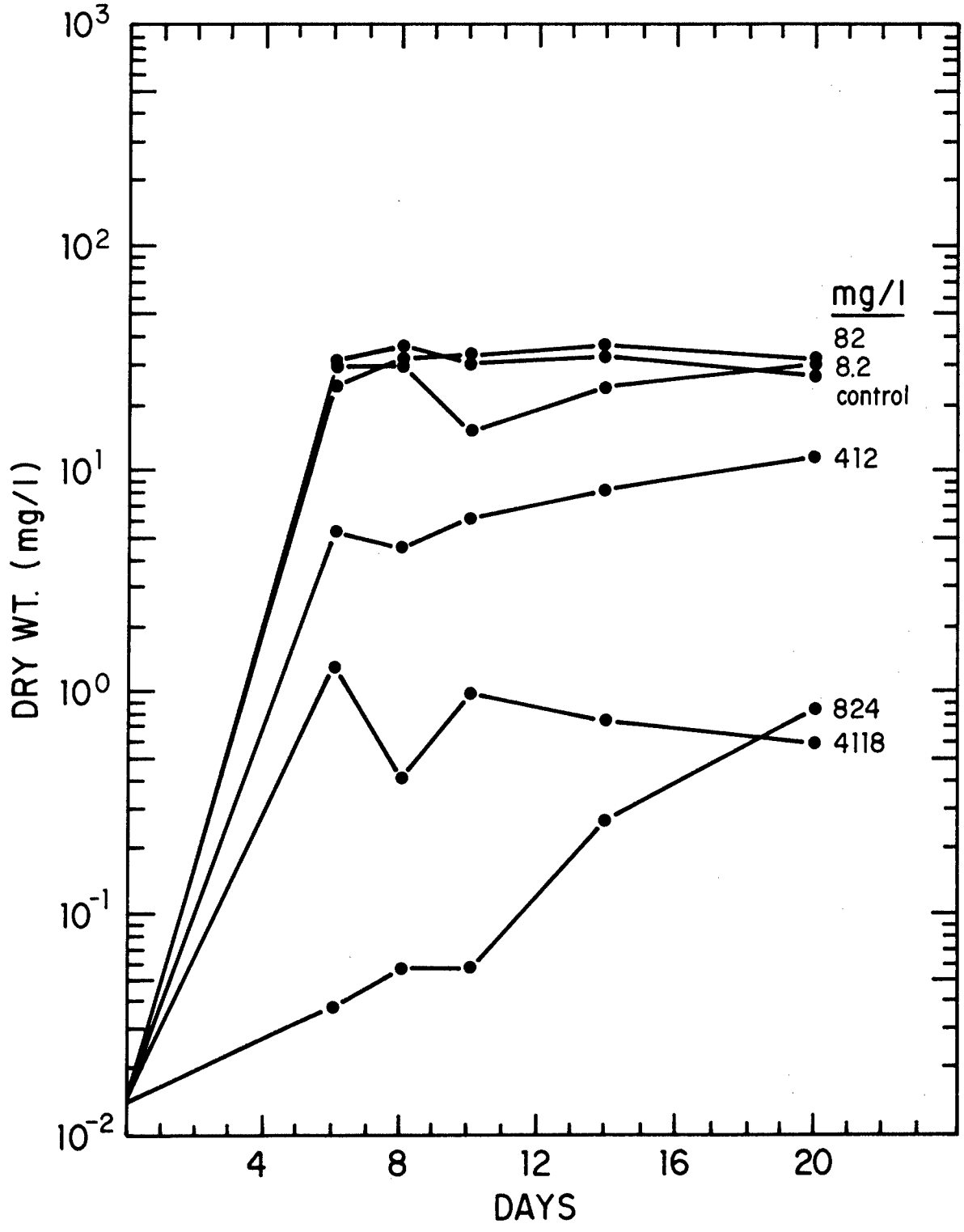

Figure 4. Growth of S. capricornutum (expressed as mg/ℓ dry weight) in 33% SAAM exposed to selected concentrations of JP-8

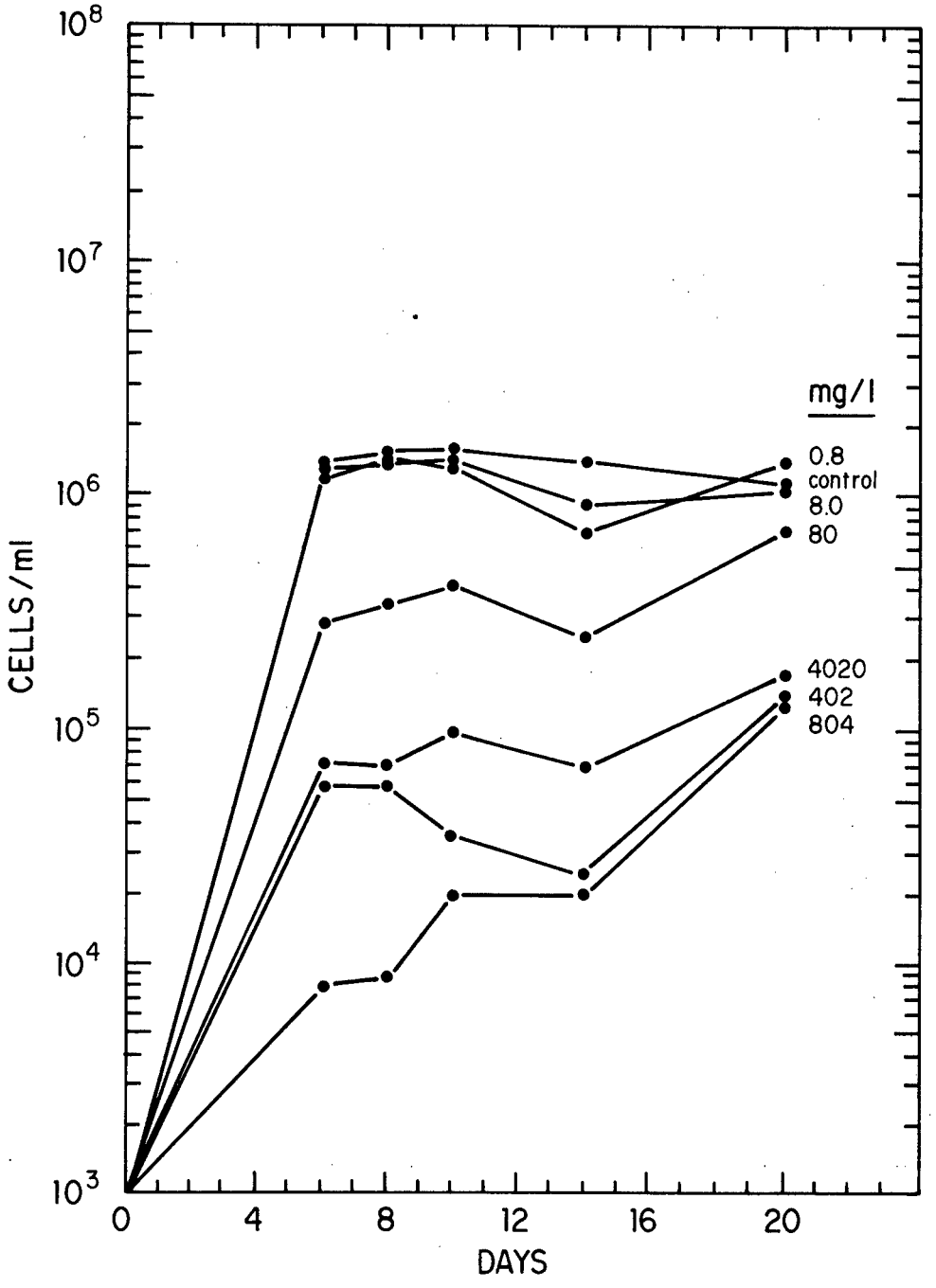

Figure 5. Growth of S. capricornutum (expressed as cells) in 33% SAAM exposed to selected concentrations of SDJP-8

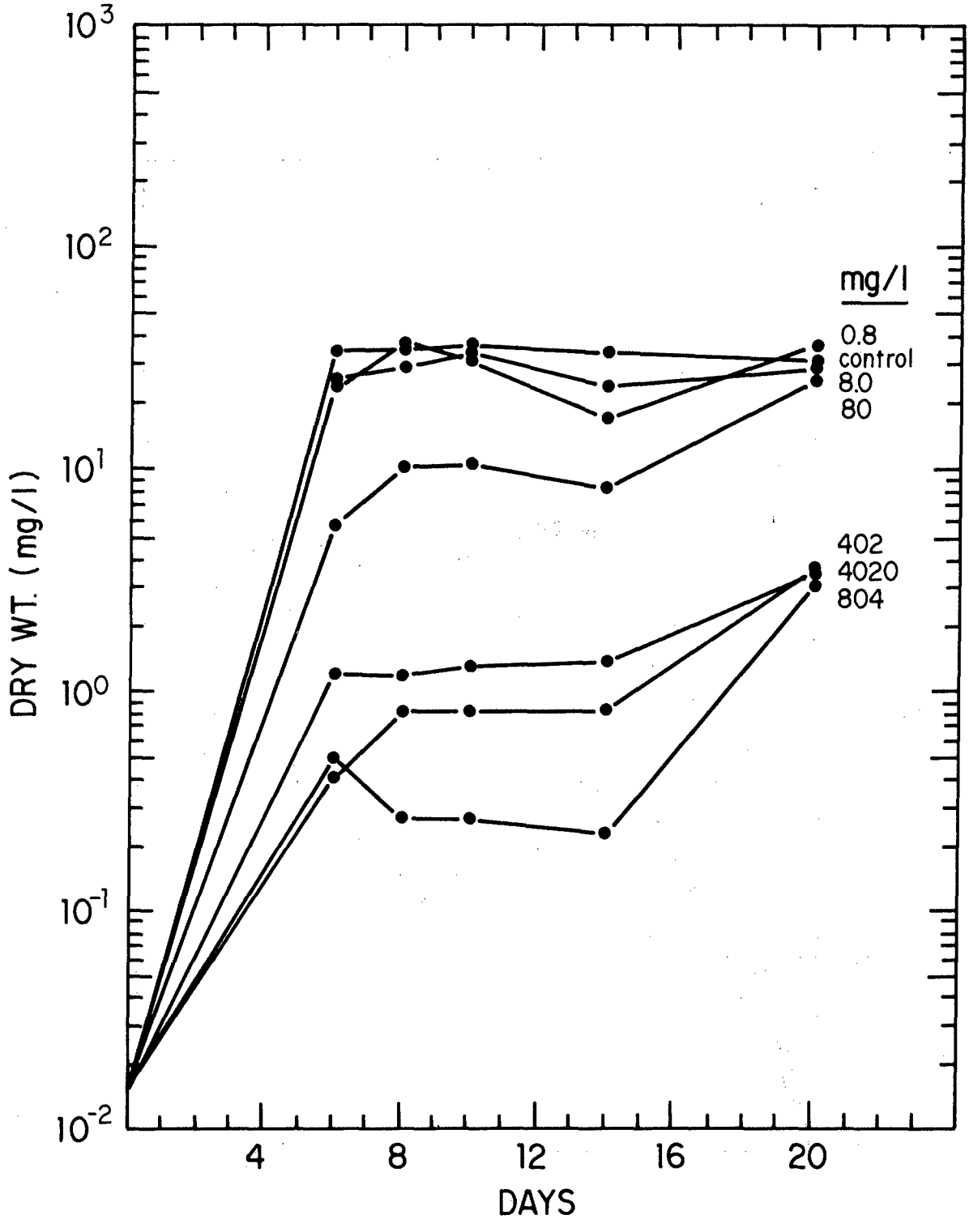

Figure 6. Growth of S. capricornutum (expressed as mg/ℓ dry weight) in 33% SAAM exposed to selected concentrations of SDJP-8

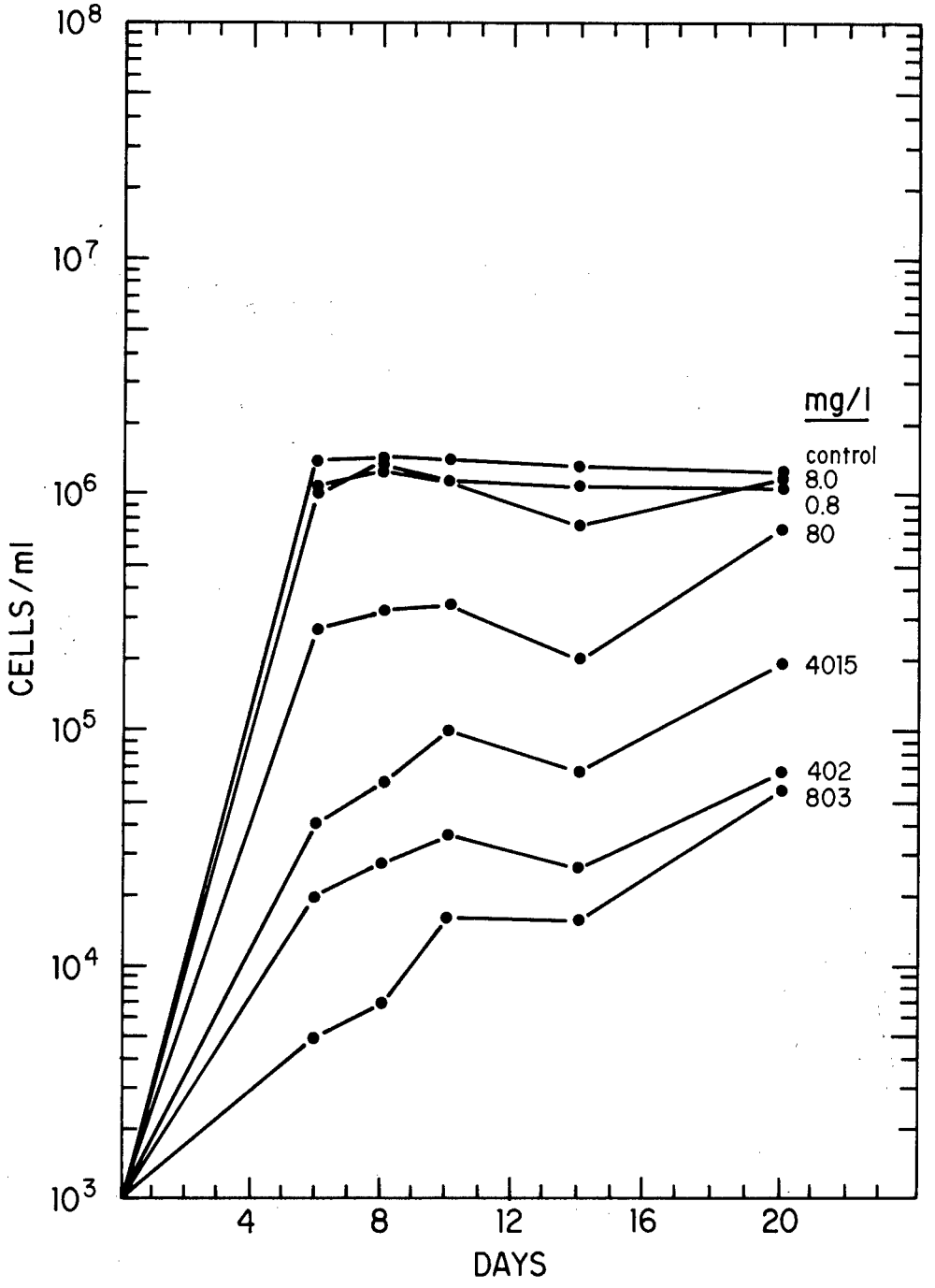

Figure 7. Growth of S. capricornutum (expressed as cells) in 33% SAAM exposed to selected concentrations of CTSDJP-8

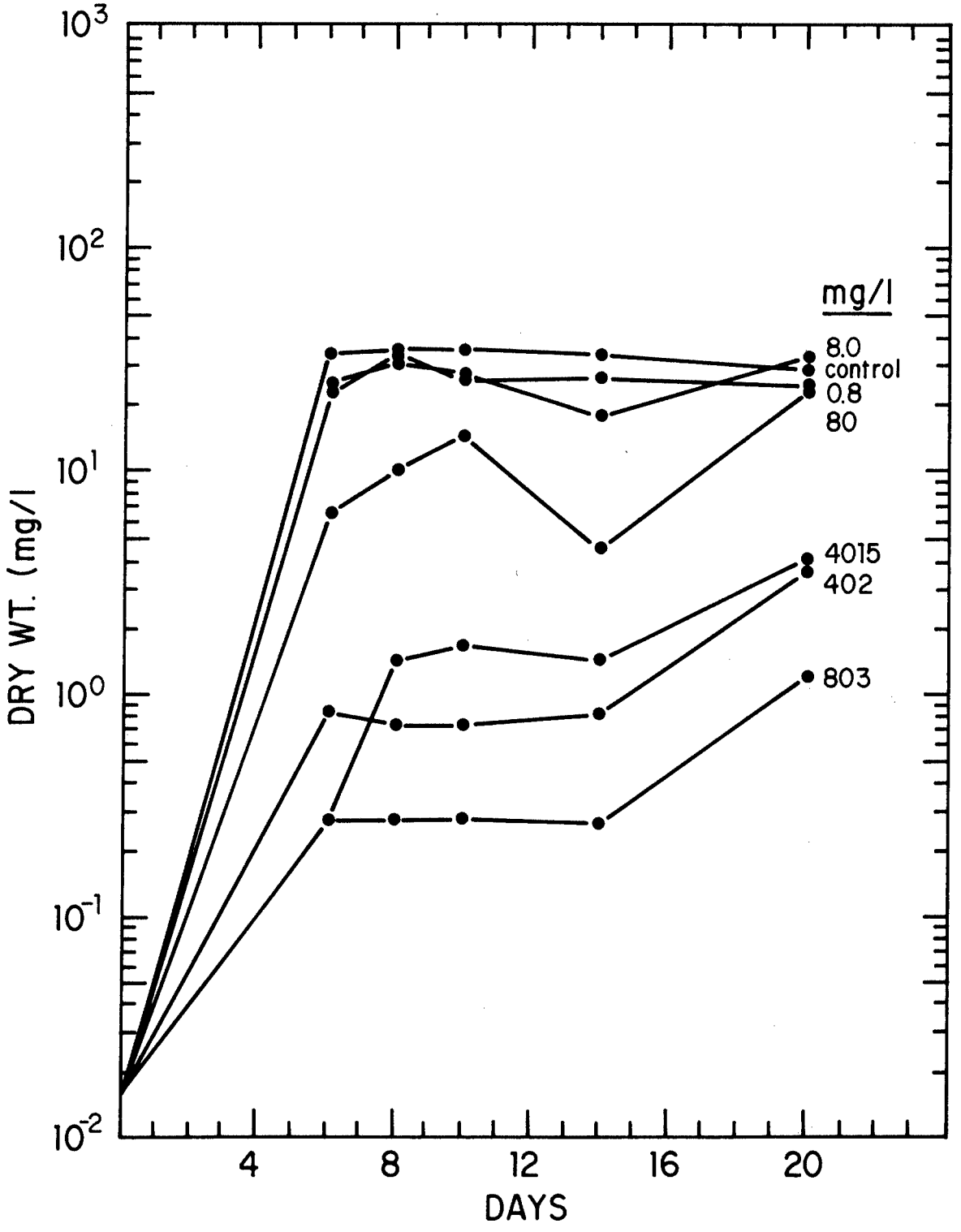

Figure 8. Growth of S. capricornutum (expressed as mg/ℓ dry weight) in 33% SAAM exposed to selected concentrations of CTSDJP-8

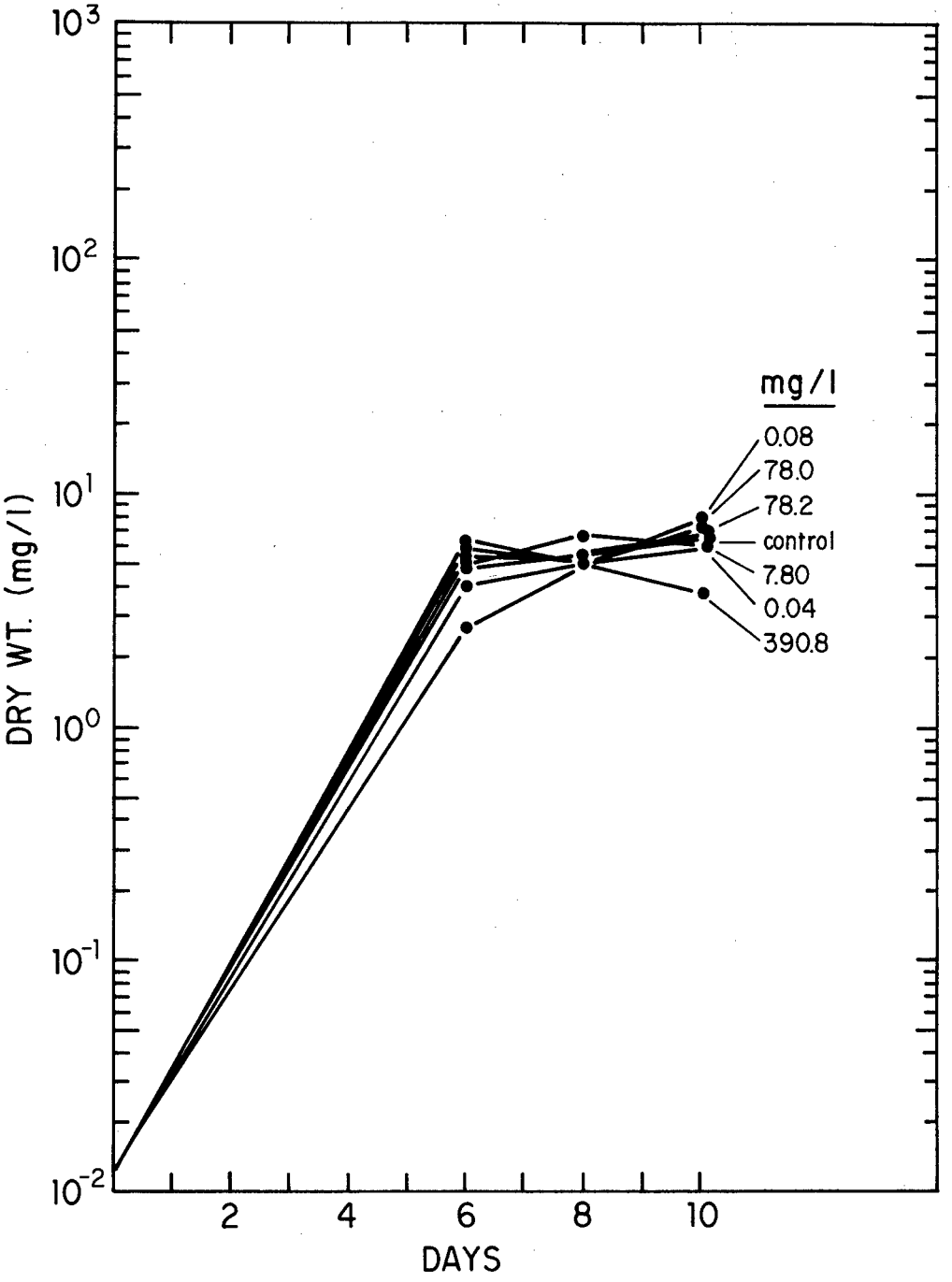

Figure 9. Growth of S. capricornutum (expressed as mg/ℓ dry weight) in 33% SAAM exposed to selected concentrations of SDJP-4

identification of specific trace metals were made to determine to what extent trace metals in the fuels might be the cause of the observed effects.

Fuel samples were analyzed for trace elements using both atomic absorption spectroscopy and neutron activation analysis (Pinta, 1978). Identified trace elements and their concentrations in JP-4, JP-8, SDJP-8, and CTSDJP-8 are presented in Table 9. Of the elements listed, those with the greatest toxic effect on algal growth are: Cr, Cu, Pb, Ni, Ti, Va, and Zn (Thomas, 1980). Four of these elements (Cu, Ni, Va, Zn) were found at such low concentrations that they cannot be regarded as sources of significant growth inhibition in algae exposed to jet fuels, unless present in an unusual metal-organic complex. In JP-8, Cr and Ti occurred at levels of 0.23 and 0.60 mg/ℓ respectively, while in JP-4, Pb occurred at 1.0 mg/ℓ. These elements were likely to produce inhibitory effects and could have been possible sources for at least part of the inhibition observed.

TABLE 9. CONCENTRATIONS OF TRACE ELEMENTS PRESENT IN JP-4, JP-8, SDJP-8,AND CTSDJP-8 OBTAINED BY EMISSION SPECTROSCOPY (ES) AND/OR NEUTRON ACTIVATION (NAA)

(Results expressed in mg/ℓ, ND = not detectable)

Element	Procedure	JP-4	JP-8	SDJP-8	CTSDJP-8
Aluminum (Al)	ES	0.15	0.22	0.34	0.49
Antimony (Sb)	ES	ND	trace	ND	ND
Boron (B)	ES	ND	0.01	ND	ND
Bromine (Br)	NAA	2.72	0.12	0.05	0.06
Calcium (Ca)	ES	0.13	0.09	0.22	0.20
Chromium (Cr)	ES	ND	0.23	ND	ND
Chlorine (Cl)	NAA	5.44	0.86	0.62	1.00
Copper (Cu)	ES	0.037	0.026	0.039	0.013
Iron (Fe)	ES	0.15	0.13	0.25	0.15
Lead (Pb)	ES	1.00	0.098	ND	ND
Magnesium (Mg)	ES	0.11	0.044	0.63	0.13
Nickel (Ni)	ES	0.087	0.080	ND	ND
Phosphorus (P)	ES	ND	2.20	ND	ND
Silicon (Si)	ES	4.10	0.34	2.90	1.30
Sodium (Na)	NAA	1360.00	6075.00	ND	ND
Tin (Sn)	ES	ND	0.23	ND	ND
Titanium (Ti)	ES	ND	0.60	ND	0.18
Vanadium (Va)	NAA	ND	0.006	0.001	ND
Zinc (Zn)	ES	ND	ND	ND	ND

In order to determine the possible role of these elements in the interactions between fuels and algae, each of the three elements Cr, Ti, and Pb were quantified in the algal assay medium after the introduction of fuel. For each fuel, 15 ml was added to 135 ml of deionized water in a separatory funnel to create a 10% fuel/90% water mixture. The mixture was shaken continuously at 100 rpm for a six-day period.

A sample of the WSF/E was then removed from the funnel and analyzed for Cr, Pb, and Ti, using a Perkin-Elmer Model 403 Atomic Absorption Spectrometer.

In every case, the concentrations found in the WSF/E were much lower than the concentrations in the raw fuel. For example, Cr in JP-8 was 0.23 mg/ℓ but only 0.0055 mg/ℓ in the WSF/E of that fuel. The concentration of Pb in JP-4 was 1.0 mg/ℓ but only 0.0204 mg/ℓ were measured in its WSF/E. Similarly with Ti, virtually none could be detected in the WSF/E of any of the four fuels, although 0.6 mg/ℓ was found in JP-8. Although the concentrations of Pb, Cr, and Ti in the tested jet fuels appeared to be a potential cause for the diminution in growth of S. capricornutum, investigations indicated that these metals were largely fuel-bonded, while their interaction with algae would occur almost exclusively in the WSF/E phase.

Published data in comparable studies (Chiandani, 1978; Christensen, 1979; Bartlett, 1974) as shown in Table 10, indicate that the WSF/E concentration of Cr in JP-8, Pb in JP-4 and Zn in CTSDJP-8 were respectively 0.011, 0.0205 and 0.011 mg/ℓ, and lower in all cases than the EC_{50} concentrations. Furthermore, the metal concentrations in the WSF/E were derived from a 10%/90% water mixture, while previous observations on the response of algae to test fuel exposures were obtained at fuel concentrations less than 5%, where complete inhibition occurred. These findings suggested that components other than trace elements were responsible for the total growth inhibition.

TABLE 10. Cr, Pb, AND Zn CONCENTRATIONS IN THE WSF/E OF CERTAIN FUELS

Metal	Fuel	WSF/E Concentration (mg/l)	EC_{50} (mg/l)	Source
Cr	JP-8	0.011	0.031	Chaudani (7)
Pb	JP-4	0.0205	0.140	Christensen (8)
Zn	CTSDJP-8	0.011	0.030	Bartlett (9)

BACTERIOLOGICAL INVESTIGATIONS

A screening test using standard plate counts was made to determine whether significant bacterial numbers were present in media exposed to the fuels. Petri dishes were loaded with 20 ml of heart infusion agar and seeded with growth media exposed to various fuel concentrations, after serial dilutions ranging from 1 to 1/10,000, according to Standard Methods (Standard Methods, 1975).

The screening tests were conducted in parallel with algal growth determinations using the number of cells present, and with chemical analyses to determine the amount of total carbon in solution. The tests were conducted on three different types of batch culture:

1. The stock solution (pure culture of S. capricornutum)
2. The controls immediately after seeding (no fuel added)
3. The experimental flasks with fuel added.

The plate count results for 1. and 2. are presented in Tables 11 and 12, and show that:

-- the stock solution (pure culture) included, as expected, a natural association of bacteria with S. capricornutum (about 1000 per ml).

-- when the stock solution was diluted to obtain a standard number of 10^6 cells/liter in the experimental and control flasks, approximately 50 µl of stock solution was added to 150 ml of test media. The amount of stock solution varied somewhat for each experiment and was calculated based on the actual stock solution count immediately prior to seeding. Assuming that the bacteria associated with the algal stock solution were about 1,000 per ml (Table 11), this meant that the 50 µl innoculation introduced 50 bacteria into 150 ml test solution compared to 1,000,000 algae in the same 150 ml. The Plate Counts in Table 12, which show less than one bacteria per ml of seeded growth medium immediately after inoculation, are therefore consistent with the low initial bacterial population expected from the results for the stock solution.

TABLE 11. STOCK SOLUTION (PURE CULTURE)
SELENASTRUM CAPRICORNUTUM

Dilution Factor:	1	1/10	1/100	1/1000	1/10,000
Average Counts:	<300	105	<30	<30	0

TABLE 12. BACTERIAL STANDARD PLATE COUNTS CONTROL FLASKS IMMEDIATELY AFTER SEEDING (NO FUEL ADDED)

Dilution Factor:	2	1	1/10	1/100	1/1000
Average Counts:	0	0	0	0	0

Table 13 summarizes the results of standard plate counts for experimental flasks exposed to jet fuels on growth day 6.

TABLE 13. SUMMARY OF BACTERIAL STANDARD PLATE COUNTS (INOCULATION ON BIOASSAY DAY 6 - COUNTING ON DAY 10)

Fuel (ppm)	Plate Count Dilution Factor				
JP-4	1	1/10	1/100	1/1000	1/10,000
5000	>>>300	>>300	>>300	>300	NT
1000	>>>300	>>300	>>300	>300	NT
500	>>300	>>300	>>300	155	43
100	>300	>>300	>>300	145	30
JP-8					
5000	>>>300	>>300	>300	>300	NT
1000	>>>300	>>300	>300	150	NT
500	>>>300	175	40	70	>30
100	>300	>>300	170	75	<30
SDJP-8					
10	0	0	0	0	0
1	125	<30	<30	0	0
0.1	60	<30	0	0	0
0.05	75	<30	0	0	0
CTSDJP-8					
10	>>300	>300	>300	65	<30
1	>300	>300	150	40	<30
0.1	>>300	>>300	>>300	70	<30

For JP-4 and JP-8, all samples indicated insignificant numbers of bacterial colonies. Staining tests showed that approximately 85% of the colonies were Gram negative. The comparison of algal cell numbers with colony counts on Day 6 (Tables 1, 2 and 13) indicates that for higher fuel concentrations, bacterial counts outnumber algae significantly. The reverse trend is observed for the lowest fuel concentrations, corresponding to the algal NOEL range. It is concluded that while JP-4 and JP-8 inhibit algal growth when present in high concentrations, they provide a non-inhibitory source of carbon for bacterial growth.

The standard plate counts for shale-derived fuels showed that for SDJP-8 the highest bacterial counts corresponded to an intermediate fuel concentration of 1 ppm, while there was no significant difference for CTSDJP-8. These results are significantly different from those obtained for the conventional JP-4 and JP-8 fuels. They suggest that the shale-derived fuels contain components which have inhibitory effects on both algae and bacteria. Table 13 also indicates that clay treatment may remove some bacterial-inhibiting compounds from SDJP-8. Additional counting on day 14 after inoculation showed no significant difference in JP-4 and JP-8 bacterial counts; for both shale-derived fuels, three additional incubation days produced a 10 to 15% increase in bacterial counts, with a great variability in colony size for any given dilution. Staining techniques showed that 80% of the colonies were Gram negative.

Bacterial samples from controls and from SAAM exposed to various concentrations of SDJP-8 and CTSDJP-8 were examined under scanning electron microscopy. Figures 10 and 11 show algal cells from control cultures not exposed to fuels. A few rod-shaped bacteria with rounded ends, ranging from 1 to 3 μm, of two different cell diameters, are present. The spherical disk-like structures in the background have not yet been positively identified. A 660 X magnification (Figure 12) of algal cells exposed to 10 ppm of SDJP-8 shows abundant debris, and spherical bacteria outnumbering rod-shaped bacteria. Figure 13 demonstrates a direct connection between rod-shaped bacteria and the test alga. For CTSDJP-8 at a concentration of 10 ppm, scanning revealed inexplicable clumps of cells (Figure 14). Compared with the control, the algal cells had stunted tips and numerous associated spherical and rod-shaped bacteria. At the lower concentration of 0.05 ppm of CTSDJP-8 (Figure 15), some algal cells were normal and arcuate while others were lysed.

Pure cultures of bacteria isolated from Standard Plate Counts inoculated with medium plus JP-4 or JP-8 were identified at the Orange County Health Department. Preliminary results indicated that all organisms isolated were of the genus Bacillus. Detailed

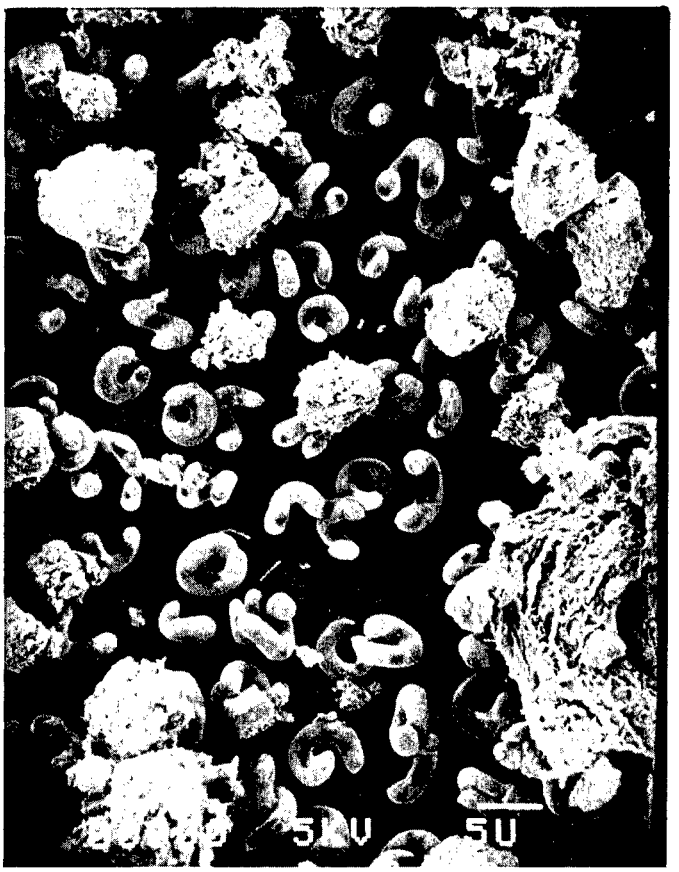

Figure 10. SEM of S. capricornutum from control stock culture (x1500)

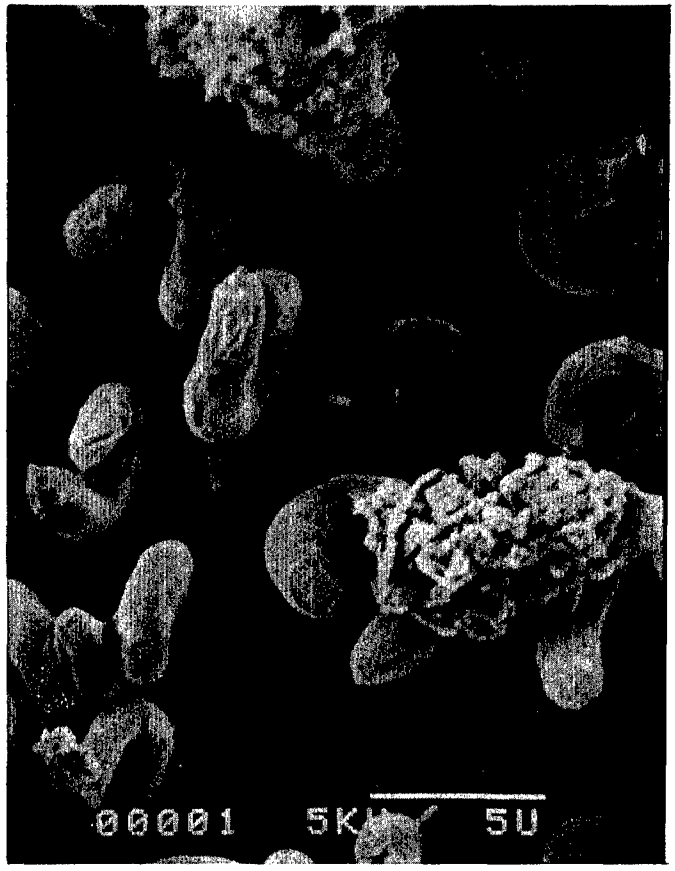

Figure 11. SEM of S. capricornutum from control stock culture (x4700)

Figure 12. SEM of S. capricornutum exposed to 10 ppm of SDJP-8 (x660)

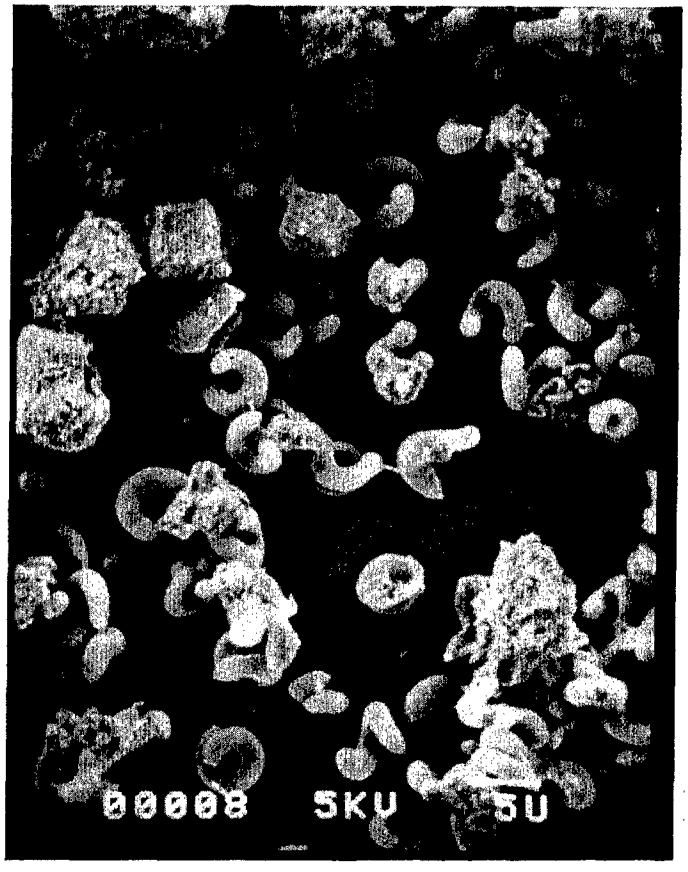

Figure 13. SEM of S. capricornutum exposed to 0.05 ppm of SDJP-8 (x1700)

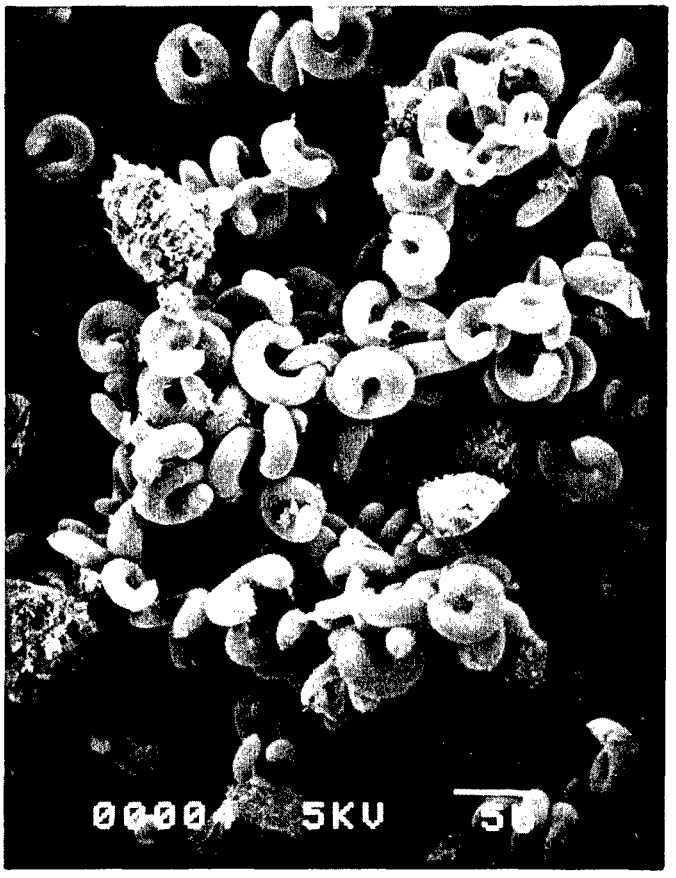

Figure 14. SEM of S. capricornutum exposed to 10 ppm of CTSDJP-8 (x1900)

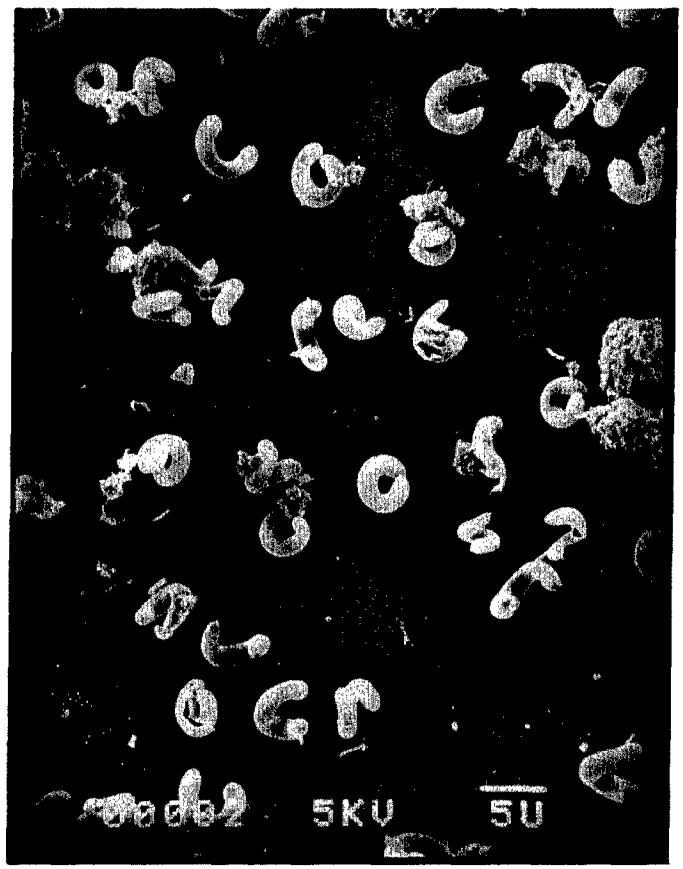

Figure 15. SEM of S. capricornutum exposed to 0.05 ppm of CTSDJP-8 (x1500)

identification of all organisms isolated could not be undertaken within the time frame/budget of the present contract, but it was ascertained that the most frequent organism encountered was identifiable as Bacillus cereus var. mycoides. Such bacteria are usually described as being rod-shaped, with rounded ends, 3.0 to 5.0 µm in length and 0.2 to 1.2 µm in breadth, and occurring frequently in chains. Colonies on agar are thin and grayish, flat and irregular in outline. The temperature optimum for the variety is about 30°C, and it has a wide distribution in soil.

Table 14 shows the TC increase in different fuel concentrations in 33% SAAM with and without algae and bacteria added. When comparing algal and bacterial counts with Table 14, results suggest the following:

TABLE 14. TOTAL CARBON INCREASE IN DIFFERENT CONCENTRATIONS OF FUEL IN 33% SAAM RATIOS FOR JP-4 AND JP-8, WITH AND WITHOUT ALGAE AT 100 RPM

	Growth medium with algae & bacteria			Growth medium without algae & bacteria		
JP-4 (ppm)	Day 6	Day 8	Day 10	Day 6	Day 8	Day 10
5000	18.5	21	23	37*	64*	LA
1000	11	12.5	11	13.5	11	12
500	14	17	21	9.5	7	6.5
100	23.5	30	34	6	7.5	10
JP-8 (ppm)						
5000	21.5	25.5	32.5	14	18.5	17.5
1000	10.5	13	13.5	14	12	12.5
500	12.5	16	18	9	13	7.5
100	21	30.5	31	6.5	6	7

*Set of values out of range LA = Laboratory accident

-- For 100 ppm concentrations of JP-4 and JP-8 with algae, TC levels are similar to those of the controls; such observation is expected since a fuel concentration of 100 ppm is close to the NOEL.

-- For 5000 and 1000 ppm concentrations of JP-4 and JP-8 with algae, the lower amount of measured TC is most likely due to the drastic decrease in the algal population, as shown by cell volume values (Tables 1 and 2).

-- For 500 ppm concentrations, bacterial counts indicate significant populations, and the recorded TC increase could result from a combination of at least two factors:

- o greater amounts of fuel in emulsion/solution
- o carbon metabolization by bacteria

-- Comparison of TC values of growth medium with and without algae and associate bacteria indicate the preponderant effects of bacteria at highest fuel concentrations and algae at lowest fuel concentrations.

BIOASSAYS OF A COMPARISON MIXTURE

Batch assays were completed to determine the toxicity of a "Comparison Mixture" (CM) for JP-4. The mixture contains 15 individual compounds from the four major classes of compound present in conventional and synthetic fuels. The composition, which was selected by the Air Force Aerospace Medical Research Laboratory, is shown in Table 15. One of the main objectives of investigating the effect of this comparison mixture was to determine if the standard algal assay could be used to identify the relative toxicity of individual compounds or groups of compounds in a fuel-like mixture. The second objective was to obtain specific response values which can then be reported and used by other laboratories as a reference for comparison of results from non-standard fuels.

TABLE 15. LABORATORY COMPARISON MIXTURE FOR JP-4
(AFESC 7 OCTOBER 1981)

	Formula Weight	Melting Point °C	Boiling Point °C	Density	g
n-Hexane	86.18	-95	69	0.659	1.0354
Cyclohexane	84.16	6.5	81	0.779	1.0224
n-Heptane	100.21	-91	98	0.684	1.0123
Methylcyclohexane	98.19	-126.3	101	0.770	1.0058
Toluene	92.14	-93	110.8	0.865	0.9985
Octane	114.23	-57	126	0.703	0.9965
Ethylcyclohexane	112.22	-111	131	0.788	1.0103
p-Xylene	106.17	12.5	138	0.866	1.0021
Cumene (isopropylbenzene)	120.20	-96	153	0.864	1.0133
1,3,5 Trimethylbenzene	120.20	-45	164.5	0.864	1.0202
Indan	118.18	-51	176	0.965	1.0057
Naphthalene	128.17	81	218	0.997	0.9986
2-Methylnaphthalene	142.20	35	241.5	1.000	1.0127
n-Tetradecane	198.40	-5.5	253	0.763	1.0034
2,3 Dimethylnaphthalene	156.23	103	269	1.003	1.01433
					15.1515

Five concentrations of the comparison mixture in 33% SAAM (1000, 500, 100, 10, and 1 ppm) were prepared with five replicates for each concentration tested. The growth response of S. capricornutum to the various CM concentrations is presented in Tables 16 and 17, indicating that slight biostimulatory effects were recorded for low CM concentrations (10 ppm from start to day 10, 100 ppm on day 10). Algal growth in media exposed to 500 ppm or more of the comparison mixture was virtually non-existent, and the results show that growth inhibition lies between 100 and 500 ppm.

TABLE 16. PARTICLE COUNT AND TOTAL CARBON OF BIOASSAYS OF THE COMPARISON MIXTURE (100 RPM)

Fuel Concent. ppm		DAY 6 Num[a]	Vol[b]	TC[c]	DAY 8 Num	Vol	TC	DAY 10 Num	Vol	TC
1	x	1021.7	74.0	23.9	1077.4	75.2	25.2	1158.6	83.0	29.0
	s	236.1	18.4	4.8	214.0	7.3	2.7	152.4	11.4	0.7
10	x	842.8	59.8	21.6	1077.4	75.2	26.6	1227.4	98.1	29.3
	s	90.2	6.3	2.2	214.0	7.3	1.1	259.7	18.4	1.5
100	x	688.9	55.0	22.6	1138.4	88.7	26.1	1043.3	91.0	27.0
	s	56.7	5.7	3.0	136.5	7.4	1.2	77.3	8.0	2.3
500	x	6.0	<.42	8.5	872.2	75.2	7.8	8.1	<.42	8.8
	s	4.8	<.42	1.8	72.3	4.5	2.3	3.3	<.42	0.6
1000	x	4.3	<.42	8.8	4.3	<.42	9.2	4.0	<.42	9.0
	s	1.1	<.42	0.9	0.6	<.42	1.4	0.8	<.42	0.7
Control	x	902.5	61.4	22.3	1162.7	84.2	25.7	1162.5	85.9	28.5
	s	201.0	12.7	0.4	28.9	1.5	0.8	231.0	17.4	0.6

(a) Total Particle Numbers (10^6 cells/l)
(b) Particle Volume (mm3/l)
(c) Total Carbon (mg/l)

* outlier
x mean of five replicates
s standard deviation

TABLE 17. SUMMARY OF THE GROWTH RESPONSE OF *S. CAPRICORNUTUM* EXPOSED TO A COMPARISON MIXTURE, EXPRESSED AS A PERCENTAGE OF CONTROLS

	6 day growth		8 day growth		10 day growth	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
	% of control by volume					
1000 ppm	<0.7		<0.5		<0.5	
500 ppm	<0.7		<0.5		<0.5	
100 ppm	89.6	9.2	89.3	5.3	105.9	9.3
10 ppm	97.4	10.3	105.3	8.8	114.2	21.4
1 ppm	120.0	30.0	89.3	8.7	108.2	13.3
control	100.0		100.0		100.0	
	% of control by population					
1000 ppm	0.5	0.1	0.4	0.1	0.3	0.1
500 ppm	0.7	0.6	0.4	0.1	0.7	0.3
100 ppm	76.3	6.3	76.0	4.7	89.2	6.6
10 ppm	93.4	10.0	97.9	11.7	105.0	22.2
1 ppm	113.2	26.2	92.7	18.4	99.1	13.0
control	100.0		100.0		100.0	

Batch assay experiments were then conducted to determine the toxicity of each of the four CM sub-groups. This experiment was conducted to provide the simultaneous evaluation of the amount of hydrocarbon going into solution/emulsion in several different concentrations of each sub-group in 33% SAAM, and to establish for reference purpose the EC_{50} and the NOEL.

Five replicates were used for each group shown in Table 18.

Group C could not be tested separately because of the high melting points of its compounds. Preliminary tests prior to the bioassay indicated that least interference would be produced by dissolving group C in group A; relative toxicities were then estimated by difference between results for group A compared with that of groups A + C. The growth response of *S. capricornutum* to various concentrations of each group is presented in Table 19, indicating means of cell numbers and volumes for each concentration tested (5 replicates).

TABLE 18. LABORATORY COMPARISON MIXTURE GROUPS
(COMPOUNDS MIXED ON A 1:1 BASIS BY WEIGHT)

Group A (straight chains)	Group B (unsaturated rings)
n-Hexane	Toluene
n-Heptane	p-Xylene
Octane	Isopropylbenzene
n-Tetradecane	1,3,5-Trimethylbenzene

Group C (double rings)	Group D (saturated rings)
Indan	Cyclohexane
Naphthalene	Methylcyclohexane
2-Methylnaphthalene	Ethylcyclohexane
2,3-Dimethylnaphthalene	

TABLE 19. PARTICLE COUNT AND TOTAL CARBON OF BIOASSAYS
(CM GROUPS, 100 RPM MIXING)

Fuel Concent. ppm		DAY 6			DAY 8			DAY 10		
		Num[a]	Vol[b]	TC[c]	Num	Vol	TC	Num	Vol	TC
	A	1131.9	79.2	25.5	1615.4	120.2	28.5	1834.5	140.0	29.8
	B	915.5	63.4	24.1	1391.3	104.7	26.4	1744.2	134.0	30.0
1	A+C	1414.5	95.1	23.3	1799.8	130.2	27.6	1736.0	126.7	31.4
	D	1046.4	74.7	24.5	1520.8	116.5	28.4	1756.9	138.2	30.4
	A	874.5	62.0	24.8	1485.7	117.5	27.5	1643.2	131.2	30.3
	B	781.0	55.8	24.5	1148.0	110.4	27.7	1663.3	132.3	30.5
10	A+C	1289.2	91.3	23.0	1825.0	136.1	28.1	1659.3	122.0	33.5
	D	879.6	62.4	22.6	1514.3	114.9	25.5	1777.6	141.6	28.5
	A	730.6	56.1	24.3	1367.1	102.0	27.5	1517.4	123.2	28.9
	B	1024.8	74.9	24.3	1598.0	125.1	26.8	1630.0	127.5	28.5
100	A+C	689.5	52.4	16.4	1553.3	126.7	26.5	1401.2	116.2	31.0
	D	1013.6	70.4	23.5	1555.4	114.4	27.5	1771.2	134.6	32.0
	A	614.6	50.8	24.7	1004.2	84.7	28.5	1002.5	89.4	30.6
	B	425.7	30.0	15.6	1210.8	93.6	22.3	1428.0	111.6	28.5
500	A+C	13.7	<0.7	11.7	13.1	<1.0	10.2	27.1	<0.8	8.8
	D	809.8	56.5	22.9	1403.8	105.9	25.7	1678.0	129.7	29.1
Control		1419.7	100.4	22.4	1705.5	126.7	25.8	1723.4	132.8	30.3

(a) NUMBER = 10^6 Cells/l. (b) VOL = mm^3/l. (c) TC = mg/l

Results from Table 19 show that at the 500 ppm level, no growth was recorded in groups A + C. Because group A showed approximately 50% of the control growth,it can be assumed that group C is the most toxic. Toxicity of group A is expected to result more from short chain components rather than n-tetradecane. At the 1 ppm level, results show for all groups that complete recovery had occurred by day 10, with no significant biostimulatory effects. Such biostimulatory effects were recorded for the CM bioassay, suggesting synergistic effects between each group.

Tables 20 and 21 summarize the results of the growth response of S. capricornutum as percentages of control.

Examination of Tables 19, 20 and 21 indicates the following ranking of increasing toxicity:

unsaturated rings < saturated rings < straight chains < double rings

CONTINUOUS CULTURES

During the middle of the 1981/1982 research period, emphasis was placed on construction and testing of a continuous culture system (as shown schematically in Figure 16).

Before proceeding with actual experiments to test fuel toxicity, it was necessary to control the exponential growth of S. capricornutum in order to reach the maximum standing crop, or ideal chemostat load for which daily cell numbers and volumes would not vary more than 5%. A first set of experiments was undertaken to test major parameters of the system such as temperature, light exposure, vessel media mixing, air flows, dissolved oxygen, pH, total carbon, phosphorus, nitrogen and trace metal concentrations.

Preliminary screening experiments conducted in February through March 1982 showed that for a fixed growth medium feed rate of 0.32 ml/minute, corresponding to 2 days residence time, the chemostats were unable to support a steady state algal population in 33% SAAM because of the gradual disappearance of nitrogen and phosphorus below their critical levels. Additional screening experiments were carried out with the objective of maintaining an appropriate balance between specific growth rate and washout of each chemostat as a function of a nutrient feed and re-residence time. During early trial runs, it was observed that phosphorus and nitrogen were growth-limiting; phosphorus reached the limit below which no cell division occurs at 0.04 mg/ℓ. Chemostats were then spiked with iron, nitrate and phosphate as soon as analysis indicated that such nutrients fell below the original concentration in 33% SAAM. Spiking produced immediate increases in cell volume and numbers, indicating

TABLE 20. SUMMARY OF THE GROWTH RESPONSE OF S. CAPRICORNUTUM EXPOSED TOCM GROUPS, EXPRESSED AS A PERCENTAGE OF CONTROLS

	Day 6		Day 8		Day 10	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
	% of control cell number					
Group A						
500 ppm	50.6	11.6	66.9	8.2	67.3	8.6
100 ppm	55.9	20.7	80.5	16.3	92.8	5.3
10 ppm	61.8	8.6	92.7	6.8	98.8	7.5
1 ppm	78.9	18.8	94.2	4.6	106.2	7.5
Group B						
500 ppm	77.2	11.4	73.9	5.5	84.2	17.8
100 ppm	74.6	8.5	98.7	4.3	96.0	7.4
10 ppm	55.6	6.5	87.1	6.3	99.7	5.5
1 ppm	63.2	20.3	82.6	10.1	100.9	4.4
Groups A+C						
500 ppm	<0.6	0.6	<0.8	0.8	<0.7	0.7
100 ppm	52.1	26.3	99.6	7.5	92.7	6.7
10 ppm	90.8	8.5	107.0	5.0	97.4	1.5
1 ppm	95.1	7.2	102.3	1.2	101.1	4.6
Group D						
500 ppm	56.3	19.6	83.6	15.7	97.7	3.6
100 ppm	70.1	17.1	90.3	9.7	101.4	4.6
10 ppm	62.2	18.3	90.7	12.0	106.6	3.8
1 ppm	74.4	16.7	91.9	2.8	104.1	3.1
Control	100.0		100.0		100.0	

TABLE 21. SUMMARY OF THE GROWTH RESPONSE OF S. CAPRICORNUTUM EXPOSED TO CM GROUPS, EXPRESSED AS A PERCENTAGE OF CONTROLS

	Day 6		Day 8		Day 10	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
	% of control cell volume					
Group A						
500 ppm	43.3	10.8	58.9	7.9	58.2	8.9
100 ppm	51.5	18.0	80.2	4.3	88.0	4.2
10 ppm	61.6	9.4	87.7	5.1	95.3	7.3
1 ppm	79.7	15.6	94.7	4.3	106.4	9.8
Group B						
500 ppm	77.2	7.8	71.1	3.7	82.9	10.2
100 ppm	72.2	7.9	93.7	4.4	84.6	7.4
10 ppm	55.0	6.4	84.9	3.0	96.5	5.9
1 ppm	64.5	18.6	81.6	16.8	101.2	7.5
Groups A+C						
500 ppm	1.0	0.6	0.7	0.2	1.6	0.6
100 ppm	46.9	21.4	87.9	5.8	81.3	8.4
10 ppm	90.9	7.5	103.3	8.4	96.2	5.5
1 ppm	99.3	6.1	101.9	1.8	100.7	9.6
Group D						
500 ppm	57.0	9.7	82.3	15.1	97.4	7.8
100 ppm	71.4	18.5	91.2	7.6	102.7	7.4
10 ppm	62.0	15.1	88.8	8.4	103.1	9.6
1 ppm	73.7	13.8	89.2	6.9	101.9	6.1
Control	100.0		100.0		100.0	

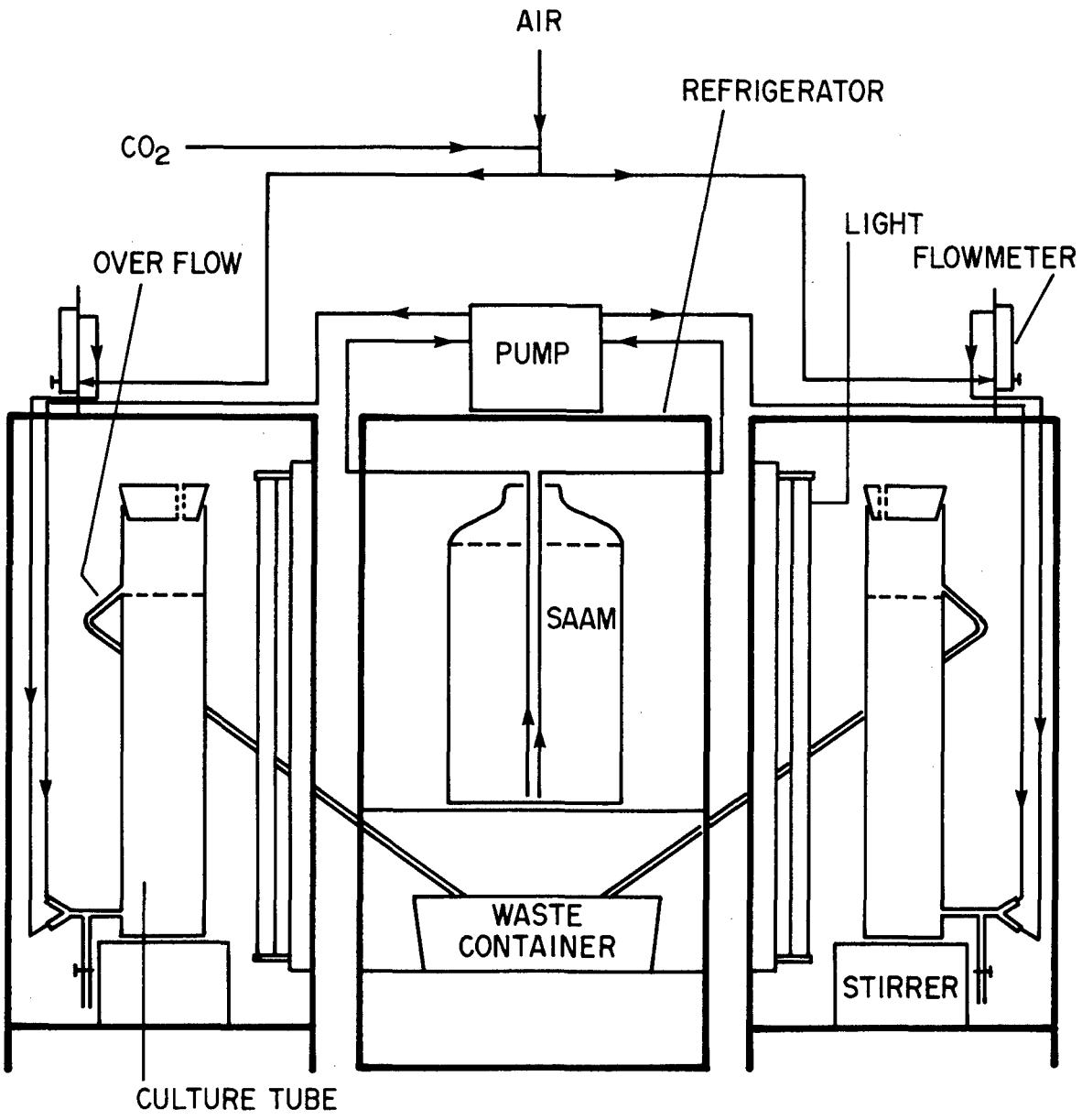

Figure 16. Continuous culture system

that the concentrations of one or more of these three elements were indeed growth-limiting. It was shown possible to "restart" overall growth in each chemostat.

The strength of the growth medium was increased to 100% SAAM with the objective of simplifying feedback control procedures and supplying additional nutrients. Following the expected initial exponential growth phase, all chemostats showed progressive achievement of the maximum standing crop over a period of 34 days. Figures 17, 18, 19 and 20 present results for the first 34 days of an ongoing continuous culture experiment.

During the same period, pH, dissolved oxygen and temperature values were not plotted, due to their consistency through day 34.

Examination of Figures 17 and 18 shows excellent correlation between cell numbers and volumes. Since no growth was recorded for chemostats 4 and 7 in the initial phase of the experiment, the two vessels were re-seeded on day 6. Overall, Figures 17 and 18 indicate that extensive growth occurred between days 3 and 8; from days 14 to 34, cell numbers and volumes oscillated between maximum and minimum values. Such variations were correlated to phosphorus and nitrogen concentrations in the chemostats. Figures 19 and 20 indicate that the lowest levels of growth-limiting nutrients occurred during the extensive growth phase. Thereafter, phosphorus and nitrogen concentrations influenced directly the algae population.

The four figures show progressive achievement of a maximum standing crop; the fragile dynamic equilibrium reached is a direct function of a successful combination of three critical parameters: feed rate, growth medium strength, and washout ratios. It is anticipated that stable and reproducible conditions will be achieved, during the last part of the project period. When steady state has been reached, the assay organisms in the chemostats will be exposed to ranges of fuels to determine and quantify the NOEL and MATC for aquatic environments under chronic release conditions.

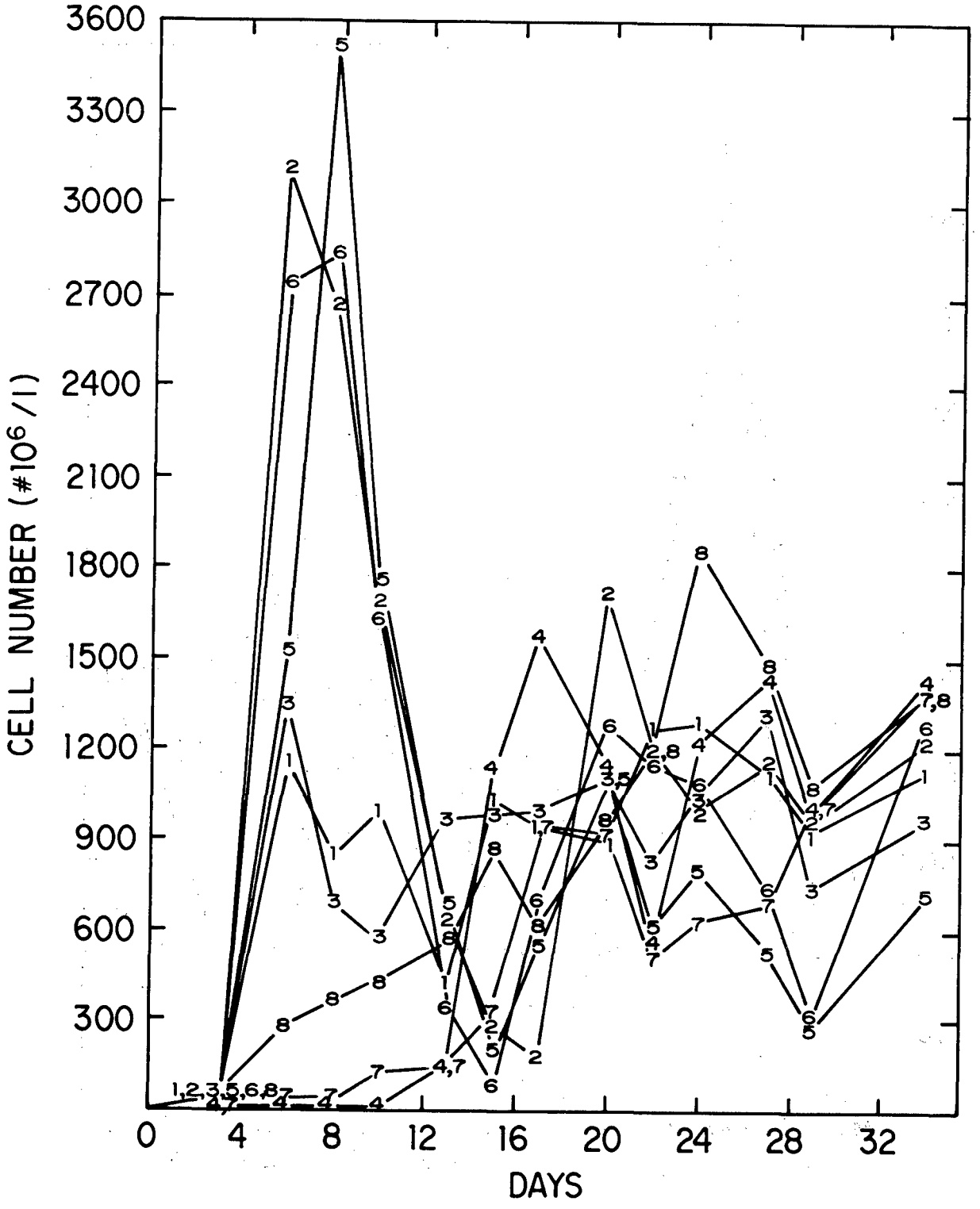

Figure 17. Growth response of S. capricornutum in eight chemostats as a function of time (all numbers expressed as 10^6 cells/ℓ)

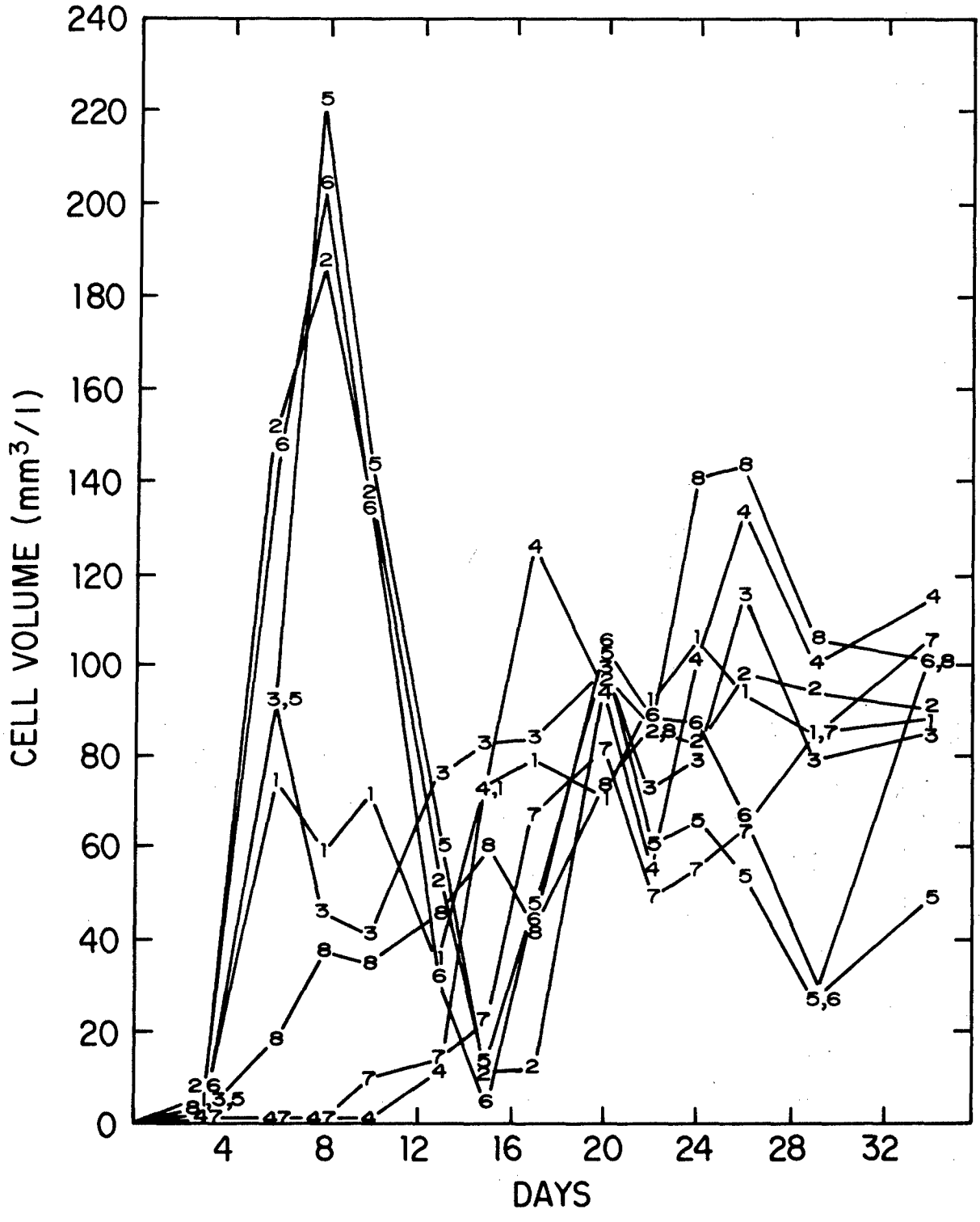

Figure 18. Growth response of S. capricornutum in eight chemostats as a function of time (all volumes expressed as mm^3/ℓ)

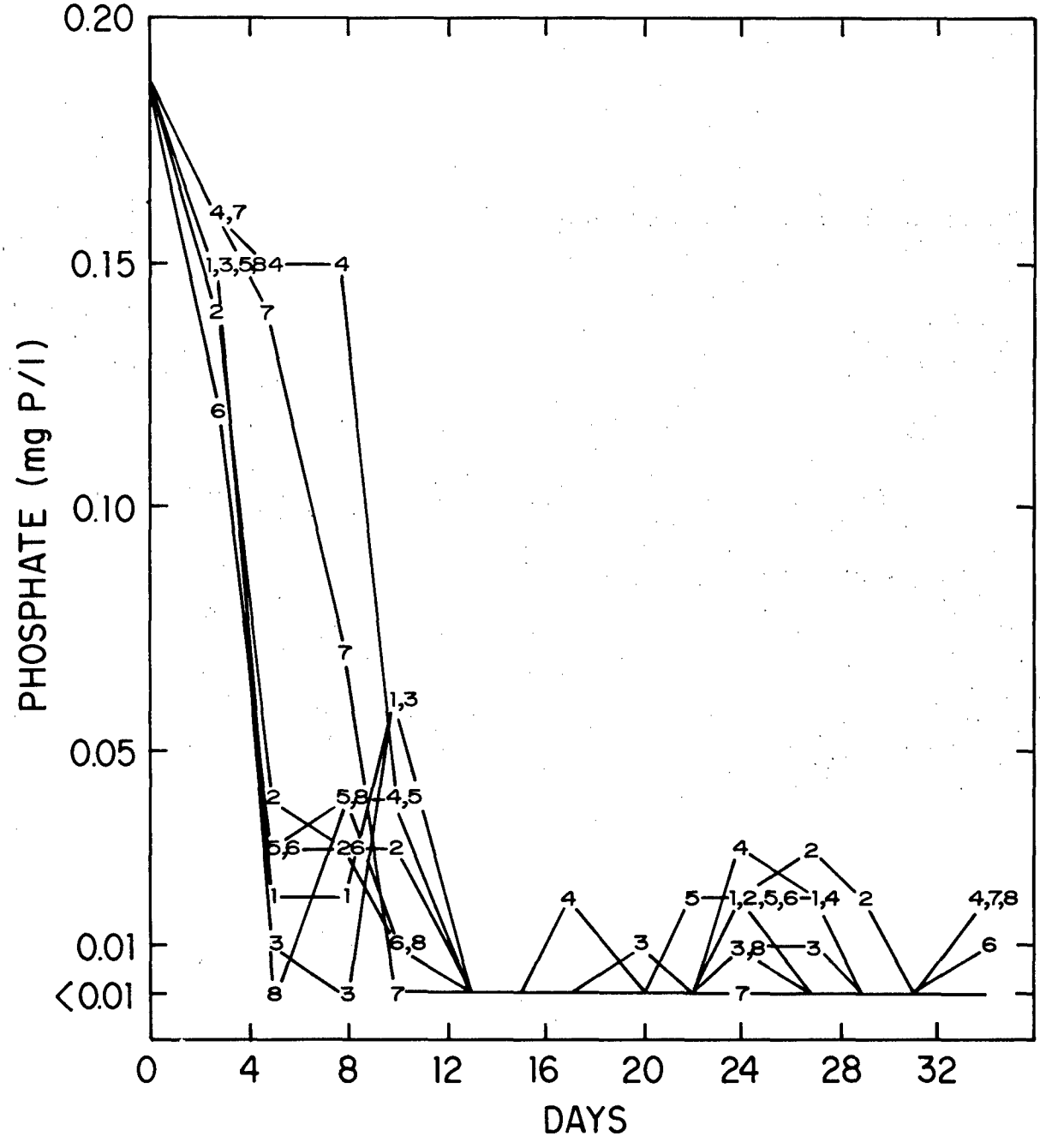

Figure 19. Phosphate concentrations in eight chemostats as a function of time

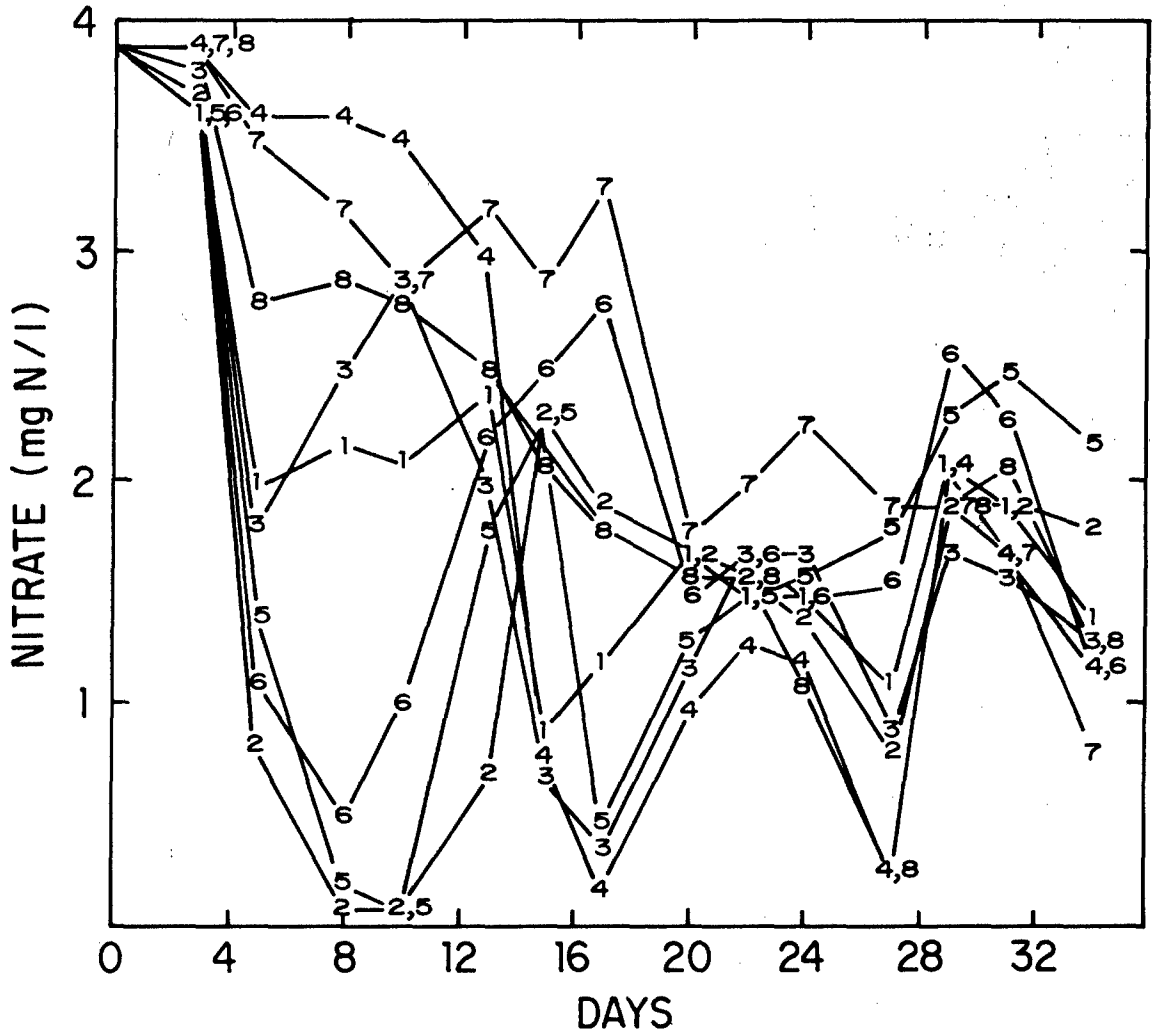

Figure 20. Nitrate concentrations in eight chemostats as a function of time

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